SEROPREVALENCE OF *CHLAMYDIA PNEUMONIAE* INFECTIONS AMONG ADULT WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASES BY ELISA AND IMMUNOFLUORESCENCE

DISSERTATION SUBMITTED TO

In partial fulfillment of the requirement for the degree of

DOCTOR OF MEDICINE IN MICROBIOLOGY

(Branch IV)M. D. (MICROBIOLOGY)

of

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CHENNAI- 600032

DEPARTMENT OF MICROBIOLOGY

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PRINCIPAL INVESTIGATOR: Dr. M.JEEVA, MBBS.,

DESIGNATION OF PRINCIPAL INVESTIGATOR: POST GRADUATE IN MICROBIOLOGY
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3. Department Research Committee Approval ☐
4. Patient Information Document and Consent Form in English and Vernacular Language ☐
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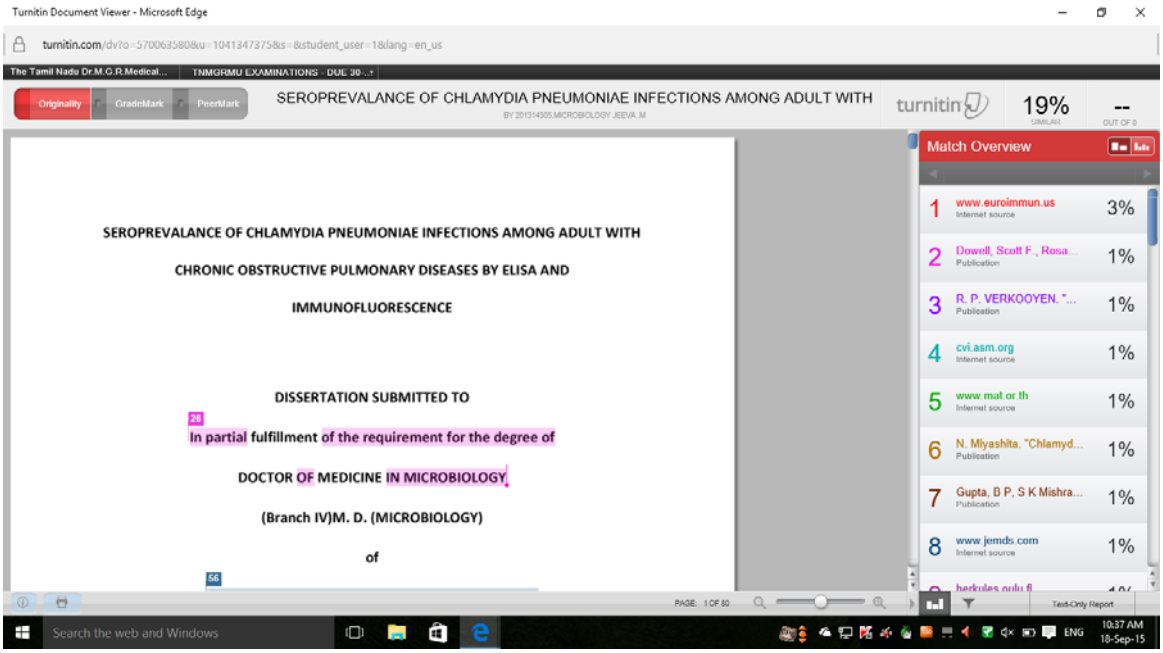
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I, **Dr. M.JEEVA** declare that, "**SEROPREVALENCE OF *CHLAMYDIA PNEUMONIAE* INFECTIONS AMONG ADULT WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASES BY ELISA AND IMMUNOFLUORESCENCE.**" I carried out this work on at the Department of Microbiology, Tirunelveli Medical College, I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree, or diploma to any other University, Board, either in India or abroad.

This is submitted to the Tamilnadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D Degree examination in Microbiology.

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LIST OF ABBREVATIONS

C.pneumoniae	-	*Chlamydia pneumoniae*
C.psittaci	-	*Chlamydia psittaci*
CDC	-	Centers For Disease Control And Prevention
COPD	-	Chronic Obstructive Pulmonary Diseases
DM	-	Diabetus Mellitus
HTN	-	Hypertension
ELISA	-	Enzyme Linked Immunosorbant Assay
IgM	-	Immunoglobulin M
IgG	-	Immunoglobulin G
IgA	-	Immunoglobulin A
IIFT	-	Indirect Immuno Fluorescence Test
LPS	-	Lipopolysaccharide
MOMP	-	Major Outer Membrane Protein
PCR	-	Polymerase Chain Reaction
PBS	-	Phosphate Buffered Solution
Th	-	T helper.

CONTENTS

ABSTRACT

Aims: To evaluate the seroprevalence of *C.pneumoniae* antibodies and its relation to the possible association of acute and chronic infection.

Material / Methods: 50 adults with COPD were tested for serum *C. pneumoniae* antibodies using the IIFT test and ELISA during their hospitalization. Serological diagnosis of acute and chronic *C. pneumoniae* infection was determined and sensitivities, specificities and predictive values of both methods were evaluated

Results: 46 patients (92%) had serological evidence of *C. pneumoniae* infection. Sensitivity and negative predictive value of ELISA for the diagnosis of *C. pneumoniae* infection was low when IIFT was used as the gold standard method. Chronic *C.pneumoniae* infection was more common in COPD adult patients.

Conclusion: *C. pneumoniae* infection occurs frequently in patients with COPD. Immunofluorescence test (IIFT/MIF) is the preferred method for monitoring the presence of antibody to this organism. The possible role of chronic *C. pneumoniae* infection in COPD progression needs further investigation.

Key words: *Chlamydia pneumoniae*. COPD. IIFT. ELISA.

1. INTRODUCTION

Chlamydia pneumoniae is a major pathogen causing respiratory tract infection. *C.pneumoniae* involves a wide spectrum of respiratory diseases including the upper respiratory tract infections like pharyngitis, sinusitis, and otitis; also causes lower respiratory diseases, such as acute or chronic bronchitis, chronic obstructive airway diseases and pneumonia. It produces infections in both immunocompetent and immunocompromised host[1,2]. *C.pneumoniae* infections have strong possible association with acute exacerbation of asthma and chronic obstructive pulmonary diseases (COPD)[3, 4].

C.pneumoniae comes under the family *Chlamydiaceae.* It was first described in the year 1986 by Grayston and coworkers. It is an obligate, gram-negative intracellular bacterium. This is the third species of *Chlamydia,* next to *C.trachomatis, C.psittaci*.

It has a unique complex biphasic multiplication cycle characterized by conversion of the infectious but non-replicating elementary body to the non-infectious, replicating reticulate body. It produces intracytoplasmic inclusions that lack glycogen.

The incubation period of *C.pneumoniae* infection is 3-4 weeks. It can be transmitted from person to person by droplet nuclei and close personal contact. It appears to be an exclusive human pathogen. No animal reservoir has been identified.

Most of the respiratory infections caused by *C.pneumoniae* are asymptomatic and remains as subclinical infection. There is evidence that *C.pneumoniae* often establishes persistent infections and persistence has been observed in blood monocytes[5].

They colonize the epithelial cells of the respiratory tract and promote inflammation via the formation of cytokines, such as interleukin-8 and tumour necrosis factor-alpha, contributes to disease progression, and is associated with severe COPD[6-8]. It induces Th2 type immune response and may lead to chronic infection.

In chronic infection it results in serious sequelae like frequent reinfections will occur in *Chlamydia,* so that inflammatory reactions are repeatedly triggered.

C.pneumoniae infection has worldwide distribution, with a seroprevalence of 40-90% in various populations[9]. It has been associated with both endemic and epidemic occurrences of acute respiratory diseases and responsible for 6-20% of all community acquired pneumonias[9]. The seroprevalence is more in the elderly population.

For laboratory diagnosis of *C.pneumoniae* CDC recommends the following methods[10]

- Cell culture
- Serology
- PCR

As the cell cultures for diagnosis *C.pneumoniae* are expensive, time consuming, labor intensive and require a specialized laboratory, the clinical

usefulness is quite restricted. For cell culture HEP -2 or HL cells in 6, 12, 24 or 96 wells are used. It needs PCR for confirmation. An average of ≥1 inclusion per culture well should be considered a presumptive positive culture.

Currently serology is the routine diagnostic technique for detecting recent *C.pneumoniae* infections.

Immunofluorescence test (MIF)/IIFT: Microimmunofluorescence is the standard serological technique. Chlamydia elementary bodies (EBs) are used as antigen in MIF test. *Chlamydia* elementary body is infective form. This MIF/IIFT test is mainly useful for the species specific diagnosis and can differentiate between IgM, IgG and IgA antibodies[9].

IgM antibody appers 2-3 weeks after the onset of illness and generally, after 2-6 months the IgM will not be detectable. IgG antibody response appears 6-8 weeks after the illness. IgG antibodies indicate either chronic or repeated infection.

Acute infection of *C.pneumoniae* is defined as, a 4-fold rise in IgG or IgM titer of ≥16 and past exposure is indicated by an IgG titer of ≥16. Elevated IgA titers are main indicators of persisting infection.

ELISA was developed to defeat the disadvantages of MIF. It requires less time, more objective, easier to standardize, less expensive and can be used for large scale of population[11]. In ELISA lipopolysaccaride is used as an antigen. It can detect IgM,IgG and IgA alone or in combination.

But both MIF and ELISA can produce false positivity due to their cross reaction with other species of *Chlamydia*.

In complement fixation an enriched lipopolysaccharide (LPS) is used as *chlamydial* antigen. This has been used for many years to detect acute infections by *C.pneumoniae* and *C.psittaci.* But the sensitivity has been very low, especially in reinfection[12]. Also complement fixation assays are technically difficult. Information about the immunoglobulin classes involved in the reaction will not be detectable by complement fixation[13].

Nested PCR is more sensitive. Disadvantage of this PCR is increased risk of contamination, reamplification of the products, time consuming nature and high cost (CDC). Other PCRs are multiplex PCR, touchdown PCR, hybridization probe methods and fluorescent probe- based assays (CDC).

The fluorescent probe based assays have the advantages of closed system. So, it avoids contamination. These assays are more sensitive and more specific than single step PCR.

C.pneumoniae is sensitive to macrolides like erythromycin, azithromycin, tetracycline, but resistant to sulphonamide groups. DNA based vaccines are produced for prevention of *C.pneumoniae* infections, but only offer partial protection or no protection[14].

The seroprevalence of *C.pneumoniae* is increasing, hence in the present study we determined the seroprevalence of *C.pneumoniae* specific IgM and IgG among adult COPD patients group by the 2 serological methods i.e ELISA and IIFT and to evaluate the performance of ELISA with IIFT as gold standard.

Aims & objectives

2. AIMS AND OBJECTIVES

- To detect *Chlamydia pneumoniae* specific IgM and IgG antibodies by indirect immunoflurescence test (IIFT).
- To detect *Chlamydia pneumoniae* specific IgM and IgG antibodies by enzyme linked immunosorbant assay (ELISA).
- Comparison and evaluation of these two diagnostic methods for acute and chronic diagnosis of *Chlamydia pneumoniae* infection.

Review of literature

3. REVIEW OF LITERATURE

3.1 History

In the initial periods because of their obligate intracellular requirements, *chlamydial* species were believed to be viruses. *Chlamydia pneumoniae* was first isolated from the eye of a child, in Taiwan during the trachoma vaccine trial. Then it was named as TW-183[15]. Under electron microscopic examination Chlamydiae has DNA and RNA, as well as cell wall structures[16]. *Chlamydia pneumoniae* bacterium was described in 1965. AR-39 isolate was first isolated from a student who was suffering from pharyngitis. It was first isolated in the United States and was termed as AR-39. This name was given because of its isolation from the acute respiratory infection[17].TWAR strain transmitted from human to human[17]. It is an exclusive human pathogen. No animal reservoir.

In *Chlamydia* genus, the strains are obligate intracellular parasite with unique developmental cycle, share genus specific lipopolysaccharides, complement fixation antigen. These all criteria fulfill the *Chlamydia* species. So *C.pneumoniae* was identified as a separate species in 1989 within the genus *Chlamydia*[18].

3.1.1 TWAR:

In Sweden, during 1979 to 1992 among 16 young Swedish orienteers, 14 were suffered from sudden unexpected cardiac death. Most of this death occurred during exercising. Their serum were positive for *C. pneumoniae* antibodies[19,20]. *C. pneumoniae* has high sero prevalence in humans. So these deaths are not due to

the reason, these young Swedish orienteers donot have a higher exposure to *C. pneumoniae*.

3.2 Taxonomy of *Chlamydia*

The *Chlamydiae* genus comprises nine species. Two of these were primarily human pathogens.

1) *C. trachomatis* : causing human pharyngeal, genital and ocular infections
2) *C. pneumonia* : mainly causing respiratory disease
3) *C. psittaci* : mainly in birds
4) *C. pecorum* : this is the 4 th species of this genus produce disease in cattle.
5) *C. abortus* : sheep
6) *C. felis* : cats
7) *C. suis*: pigs
8) *C. caviae* : guinea pigs
9) *C. muridarium*: mice.

3.3 Structure of *C. pneumoniae*

3.3.1 Morphology of *C. pneumoniae* (CP)

C.pneumoniae has a pear shaped elementary bodies and also has a loose outer membrane. These features morphologically distinguished *C.pneumoniae* from other *chlamydial* species[21]. All the isolates of *C. pneumoniae* doesn't have this pear shaped appearance. *C. pneumoniae* have a large periplasmic space between the outer membrane of C. *pneumoniae* elementary bodies and the cytoplasm[22].

3.3.2 Peptidoglycan layer of *C.pneumoniae*

C.pneumoniae is similar to Gram negative bacteria. During the reproductive cycle of *C.pneumoniae*, it was appeared to be surrounded by two layers. Even though *C.pneumoniae* is similar to Gram negative bacteria, the peptidoglycan layer is not present in the periplasmic space of cell membrane[23]. Penicillin binding proteins, peptide cross links analogues are present in its cell envelope. *Chlamydial* anomaly means, the growth of *Chlamydia* is sensitive to penicillin, in spite of the absence of the peptidoglycan[24]. Peptidoglycan is produced within the host cell during the bacterial growth. Peptidoglycan is necessary for reticulate body cell division[25, 26].

3.3.3 Lipopolysaccharide(LPS)

Lipopolysaccharide is exposed to the surface, like other Gram negative bacteria and present in both elementary body and reticulate body[27]. LPS is a less potent inducer of inflammation[28]. This is an appropriate marker for *Chlamydial* infections. This LPS is structurally and physiologically not identical with other *Chlamydial* species[29].

3.3.4 Major outer membrane protein(MOMP)

Major outer membrane protein was first described in 1981 in *C. trachomatis*[30]. MOMP has a molecular weight of 40 $\times 10^6$ and has four variable domains like I, II, III, IV. MOMP is the main protein for maintaining the structural intergrity of elementary bodies through the disulphide bonds crosslinking. MOMP shares several biochemical properties with *Chlamydial* porins[31]. MOMP are

multifunctional in nature. MOMP of CP was different from *C. trachomatis*. MOMP of CP is a immunogenic proteins[32].

3.3.5 Polymorphic membrane proteins

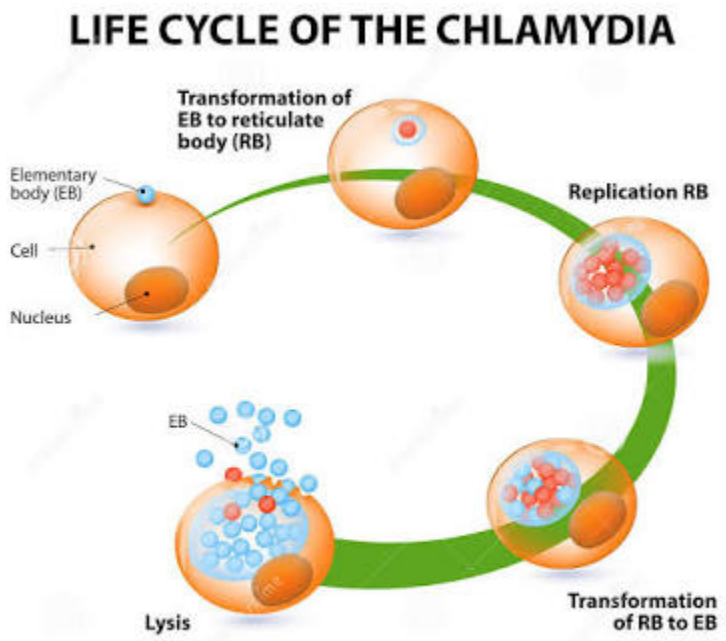
It is also a outer membrane protein, presented on the surface of *Chlamydia*[33]. This protein contains the genome, which is more significant and approximately 4% in *C.pneumoniae*[34]. In *C.pneumoniae*, 21 polymorphic membrane proteins are present[34]. All the 21 polymorphic protein genes of CP to be transcribed and produce large proteins like polymorphic protein A, B, D, E, G and H[35].

The production of proinflammatory cytokines, Interleukin- 6 , Interleukin – 8 and monocyte chemoattractant protein 1, increased by polymorphic membrane protein 20 and polymorphic membrane protein 21[36].

3.3.6 Heat shock proteins of *C.pneumoniae*

. In *chlamydial* pathogenesis, heat shock protein 10, heat shock protein 60 and heat shock protein 70 are mainly participated in the gene programming[37]. This heat shock proteins are present mainly on the outer membrane complexes of both elementary body and reticulate body[38]. In *chlamydial* infections antibodies are produced against to heat shock proteins, eg heat shock protein 60. Especially heat shock proteins 60 production causes localized inflammatory reaction in the uterine tubes.

3.4 The reproductive cycle of *C. pneumoniae*



The above picture shows various stages of *chlamydiae* multiplication cycle.

The *Chlamydiae* has a biphasic exclusive reproductive cycle and has the distinct morphological forms. It has three morphological forms

1) Elementary body
2) Reticulate body
3) Intermediate body

Elementary body is the infectious particle and metabolically inactive. This infectious elementary body has a diameter of 200 – 300 nm. Elementary body is made up of condensation of nuclear material by the bacterial histone like proteins and gene products[39, 40].

Reticulate body has a diameter of 1000- 1500 nm. It is the metabolically active form of *Chlamydia*. Reticulate body comprises homogenous internal

material and cytoplasm and made up of granular with diffuse, fibrillar nucleic acids[41].

Intermediate body formed during the transmission from reticulate body to elementary body. The duration of reproductive cycle of *Chlamydia* varies from 48-96 hours. This variation is due to the following factors like infective strain, host cells and the environmental source.

3.4.1 Attachment of elementary body to the host cell

The receptors and ligands of *chlamydia* concerned with the linkage of elementary bodies to the host cell membranes are still unclear. Most of the initial interactions of *chlamydial* strains and species are interchangeable, electrostatic interaction with glycosaminoglycan like heparin sulphate[42]. Treatment with N-glycanase decreased the attachment and inhibit the progression of disease[43,44]. Suggesting that *Chlamydial* glycans plays an important role in binding of elementary bodies into the host cell. The receptors of *Chlamydia pneumoniae*, such as mannose 6 PO_4. Retinoic acid act as an inhibitor of mannose 6 PO_4 receptors[45, 46]. *Chlamydial* surface antigen such as major outer membrane protein, outer membrane protein 2, heat shock protein 70, polymorphic protein D, polymorphic protein 2 also have a role in attachment[47].

3.4.2 Adsorption

Following attachments, elementary bodies are adsorbed into the cytoplasm of the host cell. Numerous theories proposed for the entry of elementary bodies into the host cytoplasm. One such example is parasite mediates endocytosis proposed in 1978 for the entry of *C. pneumonia* and *C. psittaci*[48]. Another entry

mechanism is through lipid raft mediated uptake[49]. Hence the adsorptive step is essential for its intracellular survival and its progression. Even though mechanism of adsorption is unclear.

3.4.3 Adsorption (Receptor mediated endocytosis)

For the adsorption into host cytoplasm, *Chlamydia* depends on active polymerization. After binding to epithelial cells, *C. pneumonia* produces pathological changes in microvilli of respiratory epithelium. Genus *Chlamydia* is responsible to regulate eukaryotic signaling pathway through phosphorylation of tyrosine residues of several proteins. Certain signaling pathways guanosine tri phosphatases, kinases are involved in the polymerization of actin[50].

3.4.4 Inclusions of *Chlamydia*

After adsorption of elementary body into the host cells, it can avoid fusion with the host cell, internalization of endosomes and lysosomes[51]. After the entry of elementary body into the host cell, it will be safe within a modified vacuole. Morphological forms of elementary bodies are smaller in size and also multiple in number. The gene expression of *Chlamydia* depends on transmission of elementary body into the perinuclear region and golgi apparatus[52].

Inclusion proteins were demonstrated in *C. psittaci*. These inclusion proteins were named from inclusion A to inclusion G. Inclusion protein A is found in *C.psittaci*. The functions of inclusion membranes are development of inclusion, avoiding fusion with lysosomes and vesicle trafficking[53].

3.4.5 Changing of elementary bodies to reticulate bodies and their proliferation

Within the host cell, elementary body differentiates to reticulate body. In the host cell, reticulate body reproduction highly shows dependence towards host cells for their energy, iron requirement and amino acids within 8 to 12 hours of infection. This elementary body changes into reticulate body by their binary fission by their host cells. Macrolides class of antibiotics blocks the development of reticulate body from elementary body[54]. Persistent forms are enlarged atypical *chlamydial* forms, which occur due to nutrient deficiency.

3.4.6 Elementary body exit from the host cell.

During the multiplication of the elementary body to reticulate body, we can detect the intermediate form of *Chlamydia*. In *Chlamydia* , after the development and multiplication of elementary body, first the inclusion will rupture, then the cell membrane ruptures or the host cell lysis will take place[55]. Then only the elementary body released by exocytosis within the host cell. This elementary body multiplication initiates new cycles.

3.4.7 Importance of Iron requirement for the reproduction of *Chlamydia*

Iron is an important requirement for most of the pathogenic bacterias[56]. But this iron uptake system is highly complicated. Iron binding siderophores act as a major source for this mechanism. It has some limitation also. Some bacteria utilize a known uptake system example *Mycobacteria.* But in *Listeria*, it has to access the cytoplasmic iron from ferritin[57].

Chlamydia uses the holo- trasferrin for the iron source during its developmental process[58]. The intracellular iron is converted from ferric to ferrous form, then it wil be transported into the cytoplasm[59]. But *Chlamydia* will escape from this early process. So in *Chlamydia* it can use the iron during the early infection endosome, throughout their reproductive cycle.

3.4.8 Persistence of *Chlamydia*

Chlamydia doesn't have a complete reproductive cycle. It needs some essential amino acids, like tryptophan for its completion of reproduction[60]. But this aminoacid is not present in *Chlamydia*. Secondly, depletion of iron requirement by the iron chelators like desferoxamine mesylate also change the *Chlamydia* infections as a persistence ones[61,62]. If the *Chlamydia* enters this state of persistence, it cant respond to the antibiotic treatment. And also it may be worse and lead to persistence even with the antibiotic treatments like macrolides[63]. This is because of repeated and chronic infections of the *Chlamydia* species.

3.5 Pathogenesis of *C.pneumoniae*

C.pneumoniae mainly affectsthe bronchial epithelial cells, impairing ciliary motility function. Then it produces inflammation via proinflammatory cytokine production.this is the main cause for increasing antibody level in above 50 years of age. *C.pneumoniae* produces three types of antibodies like, IgA, IgM and IgG.

It induces Th2 type of immune response. *C.pneumoniae* infection induces secretion of tumour necrosis factor-alpha by monocytes. It is the main cytokine of *C.pneumoniae* involved in the endothelial and epithelial expression of Interleukin-1 and adhesion molecules. Tumour necrosis factor alpha produced by the induction

of heat shock protein 60. It is mainly depend on concentration and time. Heat shock protein 60 induces production of matrix metallo proteinases by the macrophages,particularly matrix metallo protein-9.

C.pneumoniae infection also stimulates nuclear factor-kB. It is the main controlling agent for the transcriptional initiation of inflammatory genes[8]. Following are the important proinflammatory cytokines produced during *C.pneumoniae* infection.

- Tumour necrosis factor-alpha
- Interleukin-1 beta
- Chemokines like interleukin-8
- Macrophage chemotactic protein-3
- Adhesion molecules
- Inflammatory enzymes.

But the level of nuclear factor-kB activity is highly correlated with *C.pneumoniae* infection and dysfunction of lung.

3.6 Animal models

In *C.pneumoniae* animal studies mainly done in coronary artery diseases. Along with *C.pneumoniae*, some other pathogens like herpes simplex virus, cytomegalovirus, *Helicopylori* and dental pathogens can stimulate inflammatory responses and contribute to the pathogenesis of atherosclerosis. Animal models mainly studied for these agents. Based on this some positive association found only with *C.pneumoniae* and cytomegalovirus.

One study showed interaction between *C.pneumoniae* and murine cytomegalovirus in mice and cause development of chronic arterial disease. Another study about *C.pneumoniae* pathogenicity in cardiovascular disease was demonstrated in rabbits by intranasal injection with *C.pneumoniae*. In that study cholesterol-rich diet rabbits, showed a quicker development of artherosclerosis, also low density lipoprotein and apolipoprotein E deficient in mice infected with *C.pneumoniae* developed hyper chloestereolemia,then develop artherosclerosis.

3.7 Epidemiology of *Chlamydial* infection

C.pneumoniae is a common infection worldwide. About 40- 90% of people harboring this *C. pneumoniae* antibodies worldwide. This also causes community acquired pneumonia about 6-20%[9]. After the age of 6- 8years, *C. pneumoniae* antibody prevalence gradually increases. It can cause both epidemic and endemic outbreaks.

Incubation of *C. pneumoniae* is 3-4 weeks. *C. pneumoniae* is an exclusive human pathogen. So man is the one of the main reservoir for. It is transmitted by droplet nuclei or other respiratory secretions by man to man. No animal reservoir for *C. pneumonia.*

3.8 Clinical features

3.8.1 Acute manifestations

C. pneumoniae is a intracellular pathogen. It colonises in the respiratory epithelial cells and causes asymptomatic infections, remain mild or self restricted. It occurs world wide distribution with a seroprevalence up to 70%[64].This obligate intracellular bacteria can persist in the host. Persisting *C. pneumoniae* infections

are common in the respiratory tract or in atherosclerotic blood vessels[65]. In the acute infection, *C. pneumoniae* is transmitted via the respiratory secretions and droplets, from person to person[66]. The incubation period of *C.pneumoniae* is 3- 4 weeks. Following are the symptoms and signs of *C.pneumoniae.*

Common symptoms are cough, fever, fatigue, and myalgia and presented in > 40 percent. Less common symptoms are nausea, abdominal pain and presented in 39-11 percent. The uncommon symptoms are chest pain, diarrhea, exanthema, arthritis and presented in < 10 percent. In the beginning of infections, pharyngitis with hoarseness is present and also causes sinusitis, otitis media, tonsillitis, laryngitis and then developing into more typical bronchitis or pneumoniae. Prolonged cough is very common. The period from the onset of infection to resolution lasts several weeks.

3.8.2 Chronic manifestations

C. pneumoniae infection also causes some chronic manifestations. These are chronic bronchitis, bronchiectasis, chronic obstructive pulmonary diseases, bronchial asthma. Apart from respiratory infections, it also causes disorders like sarcoidosis, multiple sclerosis and reactive arthritis[67]. And also causes development of atherosclerosis, arteriosclerotic plaque formation, coronary heart disease,coronary artery stenosis and aortic valve stenosis.

In respiratory diseases of *C. pneumoniae*, smoking is the main predisposing factor. Most of the smokers will get chronic obstructive lung diseases by the age of 50years.

Clementsen *et al*[68], studies claim an etiological role of *C.pneumoniae* in asthma in adult patients. It also causes some extrapulmonary manifestations like, erythema nodosum,thyroiditis,encephalitis,and the Guillain- Barre syndrome.

3.9 Risk factors

3.9.1 Age

C.pneumoniae infections considered as a most important cause for elderly COPD patients hospitalization in all over the the world. In India, this infection affects all the age groups.

Grayston *et al*[69] studied there is a bimodal age distribution of infection with a peak at 8- 9 years and another starting at 70. In northern India Sanjay *et al*[70] studied in 10 children of persistent asthma with acute exacerbation in the age group of 5-14 years.

Allegra *et al*[71] studied in adult out patients in range of 17-54 years with a mean age group of 42 years. Based on the study Naoyuki *et al*[72] the mean age group was 67.6 years, they studied with 205 patients with stable chronic lung diseases without ARTIs.

3.9.2 Sex

Sontana *et al*[73] reported that higher prevalence of *C.pneumoniae* infections was found in males than in females, the ratio being 17:1. Marrie *et al* study revealed that the seropositivity rate is usually higher in males.

A similar study Georgios *et al*[74] revealed that males showed higher prevalence than females.

3.9.3 Association of smoking

Smoking status increases the prevalence of *C.pneumoniae* infections among COPD patients, particularly in elderly patients.

Georgios *et al*[74] studied out of 75 COPD patients, all had history of smoking. In Sontana *et al*[73] study showed that among 127 COPD patients 97 were smokers. Alyson *et al*[75] study showed that among 392 COPD patients, all had history of current or Ex smoking.

3.9.4 Association of cough

Chronic persistent cough is the one of the main symptom leading to chronic *Chlamydia* infections.

Naoyuki *et al*[76] study showed that 366 adult patients with persistent cough lasting in excess of 2 weeks. Kwang-Jun Lee *et al*[77] study showed that 137 students had cough and tested for *Chlamydial* infections by using ELISA and IIFT.

3.9.5 Association of breathlessness

Difficulty in breathing and its severity is one of the main symptoms in COPD patients.

Sanjay *et al*[70] studied seroprevalence of *Chlamydia pneumoniae* infection in children from Northern India; they divided mild persistent, moderate persistent, severe persistent asthma.

Blasi F *et al,* studied seroprevalence in 42 patients with acute exacerbation. Georgios *et al*[74] studied among 75 patients, chronic *C.pneumoniae* infection was more common in patients with moderate and severe disease than in patients with mild disease.

3.10 Laboratory diagnosis of *Chlamydia pneumoniae* infections

With respect to our serology techniques, the detection of current disease is hindered by the high prevalence of IgG in elderly patients due to repeated asymptomatic infections. Continuous cell culture remains the gold standard for demonstrating current infection. But, this technique is complex and has limited sensitivity. According to CDC, a positive result should only be declared after confirmed by another technique like PCR. Nucleic acid amplification techniques are the most sensitive to improve *C.pneumoniae* detection.(CDC)

3.10.1 Cell culture:

Cell culture is the most widely used technique. But it is a problematic and has methodological difficulties, especially from tissue samples, like vascular tissue samples. *C.pneumoniae* is an obligate Gram negative intracellular bacterium. It is very essential that *C. pneumoniae* has to be cultivated within a eukaryotic host cells. But the cell culture leads to contamination with other bacterial species eg. *Mycoplasma*. So it is very difficult to grow the *C. pneumoniae* with cell culture.

Following specimens can be taken for cell culture:

Usually *C. pneumoniae* causes respiratory tract infections. So we can collect the samples like nasopharyngeal swabs, or oropharyngeal swabs, broncho alveolar lavage and vascular tissue biopsies.

Specimen collection:

The swabs should be collected with Dacron tip, aluminum or a plastic shaft. Swabs like calcium alginate, cotton tips and wooden shafts may inhibit the growth of *C.pneumoniae*.

Transport of specimen:

All specimens should be cultured within a day (24 hours). If any necessary for transportation, it should be kept at 4°C and transported. If not able to culture on that day of collection, it should be kept at - 70°C for further processing

Processing of specimen:

Swabs taken from the transport medium, compressed along the two sides of the tubes to deliver the extra liquids. Cell lines to be used are Shell vials. We can also use HL cells in 6, 12, or 96 well tissue culture plates and HEP- 2.

Media for cell culture:

Eagle´s minimal essential medium, Iscove´s modified Dulbeceo´s medium, supplemented with 10% of fetal calf serum, Glutamine, and some non essential aminoacids, vancomycin (25µl) and amphotericin B(2µg/ml).

Then inoculate the homogenized specimens into a culture medium which contains cycloheximide. Then incubate the specimen at 35°C along with 5% CO_2 and then see the culture after 3 days.

Identification of inclusions: It should be measured by using inclusion forming units / ml. this should be done after fixation and staining with a monoclonal antibodies, like genus species and species specific antibodies.

Results: Detection of culture for *C.pneumoniae* is identification of an average of ≥one inclusions per well or tube be considered a "presumptive positive". But always culture should be confirmed by an additional test, like PCR and reported as "confirmed positive".

Quality assurance of cell culture:

Positive control: *C. pneumoniae* infected cells.

Negative control: Cells that was not infected by *C. pneumoniae*.

Naoyuki *et al*[72]**,** studied in 205 patients with stable chronic lung diseases without acute respiratory tract infections. In his study, no positive cases were found with cell culture. Kwang-Jun Lee *et al*[77], studied *C.pneumoniae* infection in an outbreak at Korea in the year of March 2004. He was examined 137 students at that outbreak. He did serum antibody test for *C. pneumoniae* using a MIF method and ELISA, and nasopharyngeal swab tests to detect the organism by specific PCR and culture. They were unable to isolate *C.pneumoniae* by cell culture.

3.10.2 Serological methods:

C.pneumoniae infection induces IgM, IgG and IgA responses that can be detected by serological methods. It provides only a retrospective diagnosis of acute infection. So, convalescent serum specimen is needed for showing fourfold increase in titer. A paired serum samples are needed for accurate diagnosis. Because single IgG titer lack clinical relevance.

After *C. pneumoniae* infections, the IgM antibodies appear first. It occurs within 2- 3 weeks of infection. Generally IgM will not be detectable after 2- 6 months of infection. Following which Ig G will be increasing and It will be more around 6 -8 weeks of infections.

Advantage of serology test

- To find out the cause in case of any outbreak
- Can detect the seroprevalence of infection

Main disadvantages of serology test

- Difficulty in collecting paired serum samples
- IgG antibody prevalence will be higher in elderly people.
- No appropriate methods for serology test
- Also reagents have high quality and are very less.

The methods used to measure *C.pneumoniae* specific antibodies vary from laboratory to laboratory. So it needs for improvement, standardization of the methods and simplification.

3.10.3 MIF/ IIFT TEST (Immunofluorescence test)

According to CDC, microimmunoflurorescence (MIF)/IIFT is the method of choice for acute *C.pneumoniae* diagnosis.(32 emerging). It is difficult to perform, requires time. It must be subjectively interpreted by an expert with a fluorescence microscope. And it also has a inter-laboratory variations.

Littman *et al*[78] demonstrated this inter laboratory variations in MIF test by compared specific *C.pneumoniae* IgG and IgA titers in 392 individuals. They reported 55% for IgG and 38% for IgA respectively.

In culture confirmed infection, absent of MIF antibodies has been reported. This is more common in young children but rare in adults.

Mainly used antigen is LPS or MOMP of infected *C.pneumoniae* elementary bodies..

- Screening can be done on various dilutions like 1:8 or 1:16. Preabsorb serum samples with anti IgG

Staining can be done with Evans blue, then counter staining with fluorescent labeled antibodies.

Results: can be read under fluorescent microscope. The recommended criteria are four fold increase or greater in Ig M antibody or IgG antibody, that is IgM is $\geq$ 1/16, IgG $\geq$ 1/ 512.

Quality assurance:

- We have to add positive, negative controls and diluted patients serum in each step.
- Ensure the titer of positive control serum for reproducibility between each step.
- Technician should be unaware about the patients/ controls and also the status of infection.

IIFT: samples or labelled antibodies are applied to the reaction fields of a reagent tray. The BIOCHIP slides are then placed into recesses of the reagent tray, where all BIOCHIPs of the slide come into contact with the fluids, and the individual reactions commence simultaneously. These BIOCHIPs are nothing but coated with *Chlamydia pneumoniae* infected cells. This procedure discussed in detail in materials and methods.

Verkooyen *et al*[9] studied 271 COPD patients showed prevalence of 72% of IgG with MIF and 53% with r DNA lipopolysaccaride ELISA.

Kwang Jun-Lee *et al*[78], in his study positive rate of IgM antibody from the acute onset was 58%. This percentage became 47% at the convalescent phase.

3.10.4 ELISA: developed to overcome the disadvantages of MIF. Because they require less time, more objective, easier to standardize. It is based on photometric reading of results and expressed in international units.(CDC)

In ELISA, *Chlamydia pneunoniae* elementary bodies with LPS are used. It is less expensive and easier to use on large scale. So, commonly used in seroepidemiological studies. Also it has a higher sensitivity and specificity. Elementary body proteins with major outer membrane proteins can also be used in ELISA shows species specific reactivity in immunoblot.

Both MIF and ELISA can produce false positive due to their cross reactivity with other species of *Chlamydia.*

Detection of human antibodies of the IgG class against *Chlamydia pneumoniae* in serum or plasma for the diagnosis of respiratory disease. (eg- COPD, bronchitis,tracheitis, and pneumonia)

Agarwal A, Chandar Y[79] studied about the correlation of chronic *C.pneumoniae* infection and bronchial asthma in serum samples of 60 adults with clinical history of asthma at 2008 by ELISA method. In their study 80% of patients had IgG anti *Chlamydial* antibody positive.

Verkooyen *et al*[9]*,* studied from 271 consecutive COPD patients with mean age of 66 and examined the prevalence of *chlamydial* IgG was 72% with the MIF and 53% with the rDNA lipopolysaccaride ELISA. He concluded that rDNA lipopolysaccaride ELISA *chlamydial* assays were most sensitive serological tool for diagnosing recent respiratory *Chlamydia* infections.

3.10.5 Complement fixation (CF)

- CF can identify high levels of antibody in patient serum acquired at intervals of 1 week apart
- It has low sensitivity for identification in re infections.

Von Hertzen *et al*[7] studied measurement of sputum antibodies in the diagnosis of acute and chronic respiratory infections associated with *C.pneumoniae* from 46 patients of COPD. They examined serum specimens, sputum, pharyngeal and nasopharyngeal swabs. In their study, sensitivity and specificity of enzyme immune assay was 88% and 95% respectively.

3.10.6 Polymerase chain reaction

It is a Nucleic acid based amplification technique for *C.pneumoniae* infection. *C.pneumoniae* can be identified in the following samples by PCR. They are (CDC)

- Respiratory specimens
- Vascular tissues
- Serum
- Pheripheral blood mononuclear cells

Some times variation can occur between PCRs. This is because of collection of serum, extraction of nucleic acids, products of amplification.

Nested PCR:

- In this PCR sensitivity rate is high
- Due to two primer sets are used and two times of amplification

Drawbacks of nested PCR:

- Contamination is more
- It will take longer time and high cost.

Multiplex PCR:

In this PCR it amplifies >one target sequence in the similar assay. This PCR are not so sensitive as compared to target PCR. For monitoring of PCR assay inhibition, internal or amplification controls may be used. We have other types of PCR also for the detection of *C.pneumoniae* infection. These are Hot- star PCR, Touchdown PCR, and Hybridization probe –based assays.

Specimen collection and processing of PCR:

In a one ml tube containing the transport medium, then centrifuge the tube along with the medium, the sediment (pellet) should be used for DNA extraction. Vascular or other tissue specimens should be cut into smaller pieces and then do the DNA extraction.

Quality control for PCR: All the reagents and samples taken with clean pipettes.

3.11 Treatment of *C.pneumoniae*:

Most widely used antibiotics are macrolides for *C.pneumoniae* infection. *C.pneumoniae* is sensitive to azithromycin, tetracycline, doxycyclin quinolones, and rifamycins. Because of their anti-inflammatory and antimicrobial properties,

macrolides are effective for acute *C.pneumoniae* infections. These have 80% efficacy in respiratory tract infections by *C.pneumoniae*. But, the persistence of *C.pneumoniae* not related to the development of resistance to the antibiotics. The possible explanation may be that the dosage duration and pharmokinetics of the treatments were not optimal.

Blasi *et al*[8], study showed that a group of 42 patients treated with azithromycin for 6 weeks, acute exacerbation of *C.pneumoniae*. Then the serum was tested for *C.pneumoniae* by serological methods showed clearance of infection after treatment.

For atherosclerosis patients treated with azithromycin, roxithromycin, clarithromycin.

3.12 Vaccines for *C.pneumoniae*:

DNA based vaccines are available for *C.pneumoniae*. Immunogenic preparations of these vaccines induce antibody production in animals, offer only partial or no protection. It is capable of inducing Th1 type immune response. Both live and attenuated microorganisms are not desirable; they may produce a pathological state.

Materials and methods

4. MATERIALS AND METHODS

The present study was conducted at the Department of Microbiology, Tirunelveli Medical College, Tirunelveli from January 2015 to June 2015 to evaluate the efficiency of Anti-*Chlamydia pneumoniae* ELISA IgM and IgG antibodies detection and also to compare its performance against Anti-*Chlamydia pneumoniae* IIFT for IgM and IgG antibodies respectively.

4.1 Study group

A total of 50 serum samples were collected from clinically diagnosed as chronic obstructive pulmonary diseases in adults.

4.2 Inclusion criteria

1. Cough with or without productive.
2. Phryngitis
3. Common cold
4. Difficulty in breathing
5. Fever for more than 2 weeks

4.3 Exclusion criteria

1. Paediatric age group
2. Pregnancy patients
3. Bronchiectasis

4.4 Ethical clearance

Ethical clearance was obtained from the college ethical committee before the commencement of the study.

4.5 Consent

Informed consent was obtained from reliable informants of patients who participated in the study.

4.6 Questionnaire

Symptoms regarding the onset of fever & duration, presence of other clinical features like cough with expectoration, difficulty in breathing, history of smoking and alcohol, diabetes mellitus, hypertension were recorded in the questionnaire.

History of smoking and alcohol, diabetes mellitus, hypertension, previous hospitalization was also included in the questionnaire.

4.7 Study sample

A total of 50 serum samples were collected from the study group. Around 5 ml of blood sample was collected from suspected cases in the acute phase with exacerbation of COPD. Serum was separated and collected in serum vial .The specimen was properly labeled with serial number, name of the patient and date of collection.

4.8 Storage of sample

Samples were immediately stored at -80^{o}C for ELISA and IMMUNOFLUORESCENCE.

ELISA IgM Kit

ELISA IgG Kit

Micro titer plate of ELISA IgM

Micro titer plate of ELISA IgG

METHODS

All 50 samples were tested for *Chlamydia pneumoniae* IgM and IgG antibodies by both ELISA and indirect immunofluorescence test.

4.9 Anti-*Chlamydia pneumoniae* ELISA for IgM

All the 50 samples were tested by Anti-*Chlamydia pneumoniae* IgM ELISA (EUROIMMUN)

4.9.1 Principle of the test

The test kit contains 8 breaks off microtitre strips with 12 rows of strips. Each reagent wells coated with *Chlamydia pneumoniae* antigens, containing with lipopolysaccharide and major outer membrane proteins. In the first step of reaction, diluted patient samples are incubated with the wells. IgM specific antibodies for *C.pneumoniae* will bind with these antigens, if the sample is a positive one. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti- human IgM (enzyme conjugate) catalyzing a colour reaction.

4.9.2 Contents of the test kit

IgM ELISA kit contains the following items to perform the test

- Microtiterplate wells coated with infected *C.pneumoniae* antigens. Each microplate strips containing 8 individual break-off wells in a frame with 12 strips and ready for use.
- Calibrator-(IgM,human),ready for use- dark red
- Positive control(IgM,human) ready for use-blue
- Negative control(IgM,human), ready for use-green

- Enzyme conjugate is peroxidase-labelled anti-human IgM, which is prepared from goat, reddish in colour and ready for use.
- Sample buffer-containing IgG/RF absorbent (anti-human IgG antibody preparation obtained from goat), ready for use
- Wash buffer-10x concentrate
- Chromogen/substrate solution-TMB/H_2O_2,ready for use
- Stop solution-0.5 M sulphuric acid, ready for use
- Test instruction
- Quality control certificate
- Protective foil

4.9.3 Kit storage

Don't freeze the reagents. ELISA kit was stored at +2°C to +8°C.

4.9.4 Procedure of the test

All the reagents brought to room temperature (+18°C to +25°C) just about 30 minutes before use.

4.9.5 Wash buffer preparation: The wash buffer is prepared with a ratio of 1:9, i.e. 10x concentration. Before the dilution, if any crystallization occurs in the concentrated buffer, warm the wash buffer to 37°C and mix well. The quantity necessary should be removed from the bottle by means of a clean pipette and diluted with deionised or distilled water.

4.9.6 Sample predilution

For analysis the patient samples are diluted 1:101 with sample buffer. For example, add 10µl sample to 1.0 ml sample buffer and mix up well by vortexing.

4.9.7 ELISA procedure

- Transfer 100 µl of calibrator, positive and negative controls, diluted patient samples in to individual microtiterplate wells.
- The plate was covered with protective foil and incubated for 1 hour at 37°C.
- Washed three times with diluted wash buffer
- 100µl of peroxidase-labelled anti human IgM conjugate to each well.
- The plate was covered and incubated for 30 minutes at room temperature.
- Washed three times with diluted wash buffer.
- 100µl of chromogen / substrate was pipette in to each well.
- Incubated at room temperature for 15 minutes.
- 100µl of stopping solution was pipette to all wells
- The absorbance of each well was read with in 30 minutes at a wavelength of 450 nm with a reference wavelength between 620nm and 650nm.

4.9.8 Calculations of results

By the recommendation of EOROIMMUN, The upper limit of the reference range of non infected persons was define as the extinction value of the calibrator.

Formula to calculate the ratio

Extinction of the control or patient sample / Extinction of calibrator = Ratio

4.9.9 Interpretation of results

- Ratio < 0.8 :negative
- Ratio ≥0.8 to <1.1:borderline
- ratio≥1.1: positive

Negative result: A negative serological result does not exclude an infection. Particularly in the early phase of infection, antibodies may not yet be present or are only present in such small quantities that they are not detectable.

Borderline result: a secure evaluation is not possible. If there is a clinical suspicion and a negative test result, we recommend clarification by means of other diagnostic methods or serological investigation of a follow up sample.

Positive result: It indicates that there has been contact with the pathogen in the determination of pathogen-specific IgM antibodies, polyclonal stimulation of the immune system or antibody persistence may affect the diagnostic relevance of positive findings. Significant titer increase or seroconversion in a follow-up sample taken after 7-10 days can indicate an acute infection.

4.9.10 Quality control for IgM

		Reference value	Valid range
Calibrator	O.D	0.400	>0.140
Pos.control	Ratio	2.8	1.5 -4.1
Neg.control	Ratio	0.1	0- 0.7

4.10 Anti –*Chlamydia pneumoniae* ELISA for IgG

All 50 samples were tested for detection of IgG antibodies by ELISA (EUROIMMUN kit)

4.10.1 Principle of the test

The ELISA test kit provides a semi quantitative or quantitative in vitro assay for human antibodies of the IgG class against *Chlamydia pneumoniae* in serum or plasma. The test kit contains 8 breakoff reagent wells with 12 rows (8×12=96 wells), which coated with infected *C.pneumoniae* antigen, containing lipopolysaccharide and major outer membrane protein. Diluted patient samples were added to the wells and then incubated. If specific IgG antibodies present, it will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalyzing a colour reaction.

4.10.2 The test kit contains

1. A microplate wells coated with antigens -12 × 8 strips
2. Calibrator 1,200 RU/ml -1× 2 ml
3. Calibrator 2, 20 RU/ml -1× 2 ml
4. Calibrator 3, 2 RU/ml- 1× 2 ml
5. Positive control (IgG, human) - 1× 2ml
6. Negative control- 1×2 ml
7. Enzyme conjugate, peroxidase- labelled anti –human IgG - 1× 12 ml
8. Sample buffer - 1× 100 ml
9. Wash buffer -1 × 100 ml

10. Chromogen/ substrate solution, TMB / H_2O_2 - 1× 12 ml

11. Stop solution, 0.5 M sulphuric acid -1× 12 ml

12. Protective foils -2 pieces

4.10.3 Kit storage

Anti *Chlamydia pneumoniae* IgG ELISA kit was stored at 2 - 8°C

4.10.4 Test procedure

Sample predilution

The test strip wells and other reagents was prepared and brought to room temperature.

Patient samples were diluted 1: 101 in sample buffer. Dilute 10µl serum in 1.0 ml sample buffer and mix well by vortexing.

ELISA procedure

100 µl of the calibrator 2, positive and negative controls and diluted patient samples were added in to individual microplate wells.

Microplate was covered with protective foil and incubated at 37°C for 60 minutes.

The wells were washed 3 times with diluted wash buffer solution.

100 µl of peroxidase – labelled anti human IgG enzyme conjugate were added in to appropriate wells of microplate.

The microplate was covered with protective foil and incubated at room temperature for 30 minutes.

The wells were washed three times with diluted wash buffer solution.

100µl of chromogen was pipette in to each well and incubated for 15 minutes at room temperature.

100 µl of stopping solution was pipette into each well

Photometric measurement of the colour intensity was read with in 30 minutes at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm.

4.10.5 Calculation of result

Calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator 2.

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

4.10.6 Interpretation of results

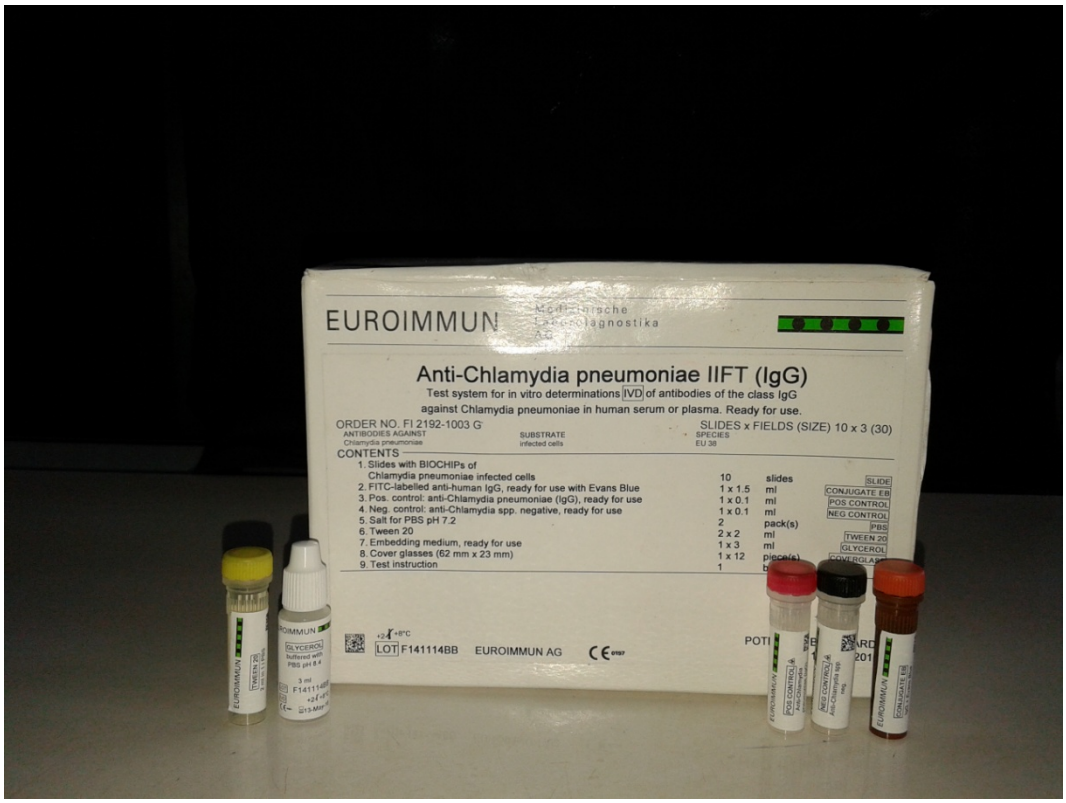
- Ratio < 0.8: negative
- Ratio ≥ 0.8 to < 1.1: borderline
- Ratio ≥ 1.1 : positive

4.10.7 Quality control for IgG

		Reference value	Valid range
Calibrator 2	O.D	0.293	> 0.140
Positive control	Ratio	3.8	2.1- 5.5
Negative control	Ratio	0.1	0- 0.7

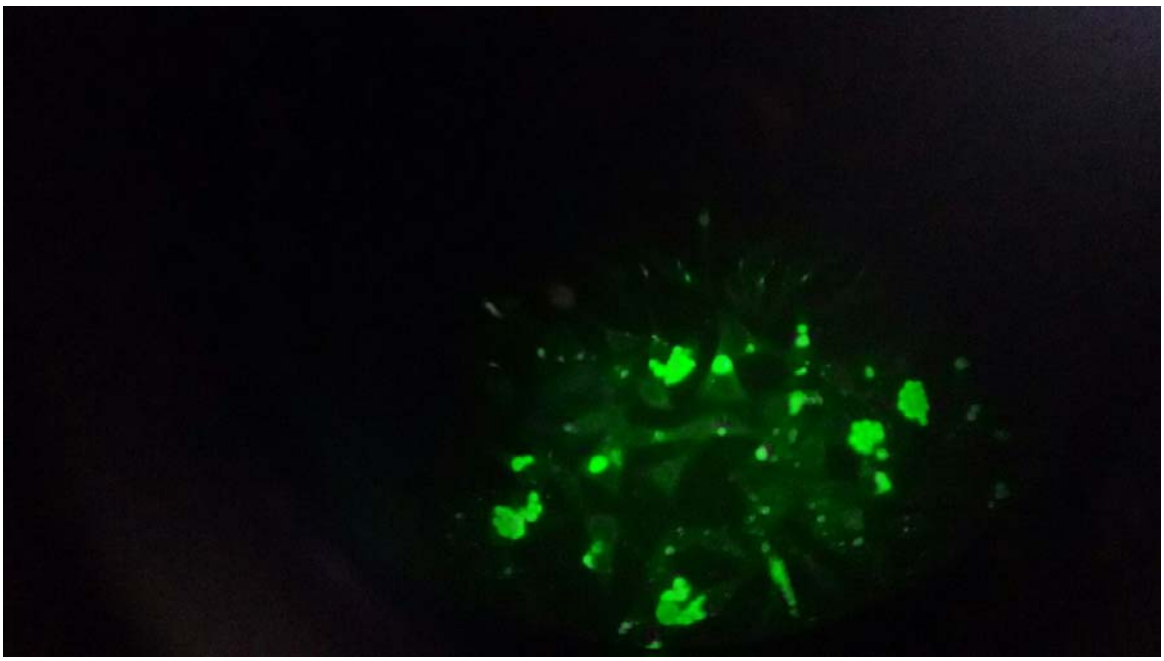
IIFT – IgM kit

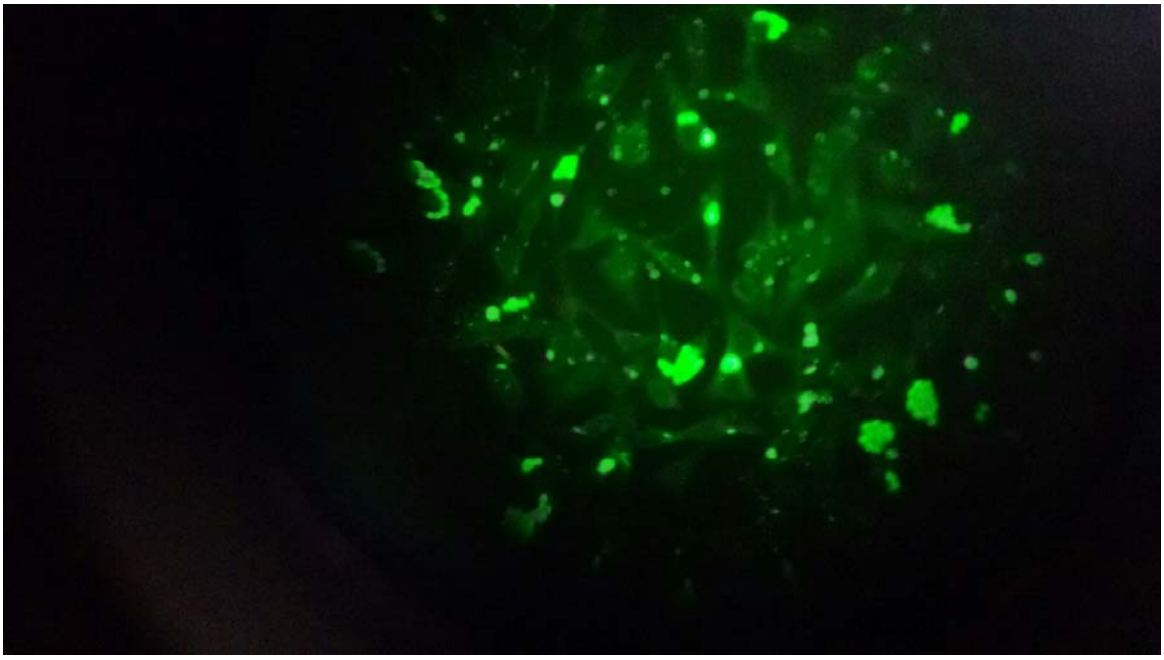
IIFT – IgG kit



Reagent tray of IIFT

Appearance of inclusion bodies in Immuno fluorescence





4.11 Anti- *Chlamydia pneumoniae* IIFT for IgM

All 50 samples were tested for detection of IgM antibodies by indirect immunofluorescence test (EUROIMMUN IIFT KIT)

4.11.1 Principle of the test

BIOCHIP coated with infected *C.pneumoniae* antigen, containing EU 38 as a substrate. Incubated with diluted patient samples. If specific IgM antibody present, it will bind to the antigens in the BIOCHIPS. Then it will stain with fluorescence conjugated antibodies. It can be seen under flurescence microscope.

4.11.2 Contents of a test kit for 30 determinations

1. 10 slides, each contains 3 BIOCHIPSs layered with *Chlamydia pneumoniae* infected cells.
2. fluorecein-labelled anti-human IgM (goat) with Evans blue- 1×1.5 ml
3. positive control: antibodies against *Chlamydia pneumonia* (IgM) human - 1×0.1 ml
4. Negative control: anti Chlamydia species. negative, human -1×0.1 ml
5. salt for Phosphate buffered solution p^H 7.2 - 2 packs
6. Tween 20- 2× 2.0 ml
7. Embedding medium(Glycerol) - 1×3 ml
8. Cover classes (62× 23 mm)- 12 pieces

4.11.3 Kit storage

The anti –*Chlamydia pneumoniae* IIFT for IgM kit was stored between + 2°C and + 8°C.

4.11.4 PBS-Tween preparation

One pack of salt for Phosphate buffered solution was dissolved in 1 liter of distilled water and mixed with 2 ml of Tween 20. Stirred for 20 minutes until homogenous. It can be stored at +2°C to +8°C, generally for 1 week.

4.11.5 Predilution of samples

Diluted the patient samples in 1: 10 dilutions.10µl of sample added to 100 µl of EUROSORB and mixed thoroughly. The mixture incubated for 15 minutes at room temperature.

Procedure for the test

- 30µl of diluted patient samples, positive, negative controls were added to each reaction field of the reagent tray without any air bubbles
- The BIOCHIPs slides were fitted in to the corresponding recesses of the reagent tray.
- Incubated for 30 minutes at room temperature
- The BIOCHIP slides were rinsed with PBS-Tween by using a beaker and then immersed in a cuvette containing PBS-Tween for at least 5 minutes.
- 25µl of fluorescein-labelled anti-human globulin added to each reaction field of a clean reagent tray
- Then BIOCHIP slides were removed from PBS-Tween, blot with a clean paper towel only on back sides and long sides of BIOCHIPs and immediately put the slides in to recesses of the reagent tray.
- Incubated for 30 minutes at room temperature

- The BIOCHIP slides were rinsed with a flush of PBS-Tween by using a beaker and immersed them in to the cuvette filled with new PBS-Tween for 5 minutes.
- Embedding medium was placed onto a cover glass-maximum 10µl per reaction field
- BIOCHIPs were removed from PBS- Tween and wiped with clean paper towel on back and all the four sides of slides.
- The BIOCHIP slides facing downwards, onto the prepared cover glass.
- Read the fluorescence with the microscope under objective 20×, 40×, with excitation filter: 450-490 nm

Fluorescence pattern (positive reaction): Antibodies against *Chlamydia pneumoniae* basis a fluorescence of the inclusion bodies located in the cytoplasm of infected cells on the test substrate. These inclusion bodies contains densely organized elementary bodies (infectious form of *Chlamydia,* diameter 300 nm) and reticular bodies (non-infectious form of *Chlamydia*, diameter 1000 nm)

4.11.6 Qualitative evaluation of results

No reaction at 1: 10 - Negative. No IgM class antibodies against *Chlamydia pneumoniae* detected in the patient sample.

Positive reaction at 1:10 – Positive. Indicates acute infection.

4.12 Anti- *Chlamydia pneumoniae* IIFT for IgG

All 50 samples were tested for detection of IgG antibodies by indirect immunofluorescence test (EUROIMMUN IIFT KIT)

4.12.1 Principle

BIOCHIP coated with infected *C.pneumoniae* antigen, containing EU 38 as a substrate. Incubated with diluted patient samples. If specific IgG antibody present, it will bind to the antigens in the BIOCHIPS. Then it will stain with fluorescence conjugated antibodies. It can be seen under flurescence microscope.

4.12.2 Contents of a test kit for 30 determinations

1. 10 slides, each contains 3 BIOCHIPSs layered with *Chlamydia pneumoniae* infected cells.
2. Fluorescein-conjugated anti-human IgG (goat) with Evans blue- 1×1.5 ml
3. 25263. Positive control: antibodies against *Chlamydia pneumoniae* (IgG) human -1×0.1 ml
4. Negative control: anti *Chlamydia* species. negative, human -1×0.1 ml
5. salt for Phosphate buffered saline p^H 7.2 - 2 packs
6. Tween 20- 2× 2.0 ml
7. Embedding medium(Glycerol) - 1×3 ml
8. Cover classes (62× 23 mm)- 12 pieces

4.12.3 Kit storage

The anti –*Chlamydia pneumoniae* IIFT for IgG kit was stored between + 2°C and + 8°C.

4.12.4 PBS-Tween preparation

One pack of salt for PBS was dissolved in 1 liter of distilled water and mixed with 2 ml of Tween 20. Stirred for 20 minutes until homogenous. It can be kept at +2°C to +8°C, usually for 1 week.

4.12.5 Predilution of samples

Diluted the patient samples in 1: 100 dilutions.10µl of sample added to 1000 µl of EUROSORB and mixed thoroughly. The mixture incubated for 15 minutes at room temperature.

Procedure for the test

- 30µl of diluted patient samples, positive, negative controls were added to each reaction field of the reagent tray without any air bubbles
- The BIOCHIPs slides were fitted in to the corresponding recesses of the reagent tray.
- Incubated for 30 minutes at room temperature
- The BIOCHIP slides were rinsed with PBS-Tween by using a beaker and then immersed in a cuvette containing PBS-Tween for at least 5 minutes.
- 25µl of fluorescein-labelled anti-human globulin added to each reaction field of a clean reagent tray
- Then BIOCHIP slides were removed from PBS-Tween, blot with a clean paper towel only on back side and long sides of BIOCHIPs and immediately put the slides in to recesses of the reagent tray..
- Incubated for 30 minutes at room temperature
- The BIOCHIP slides were rinsed with a flush of PBS-Tween by using a beaker and immersed them in to the cuvette filled with new PBS-Tween for 5 minutes.

- Embedding medium was placed onto a cover glass-maximum 10µl per reaction field
- BIOCHIPs were removed from PBS- Tween and dry back and all four sides with a paper towel
- The BIOCHIP slides facing downwards, onto the prepared cover glass.
- Read the fluorescence with the microscope under objective 20×, 40×, with excitation filter: 450-490 nm

Fluorescence pattern (positive reaction): Antibodies against *Chlamydia pneumoniae* basis a fluorescence of the inclusion bodies located in the cytoplasm of infected cells on the test substrate. These inclusion bodies contains densely organized elementary bodies (infectious form of *Chlamydia,* diameter 300 nm) and reticular bodies (non-infectious form of *Chlamydia*, diameter 1000 nm

4.12.6 Qualitative evaluation of results

No IgG class antibodies against *Chlamydia pneumoniae* detected in the patient serum- indicates negative test (at 1:100)

Positive reaction at 1:100 – Positive. Indicates previous infection.

Results

5. RESULTS

5.1 The Study Group

A total of 50 adults aged above 30 years who fulfilled the criteria of suspected COPD were analysed. This study was conducted at the Department of Microbiology, Tirunelveli Medical College Hospital, Tirunelveli over a period of 6 months from January 2015 to June 2015.

5.2 Statistical Analysis

All the results obtained were analysed statistically for their completeness, consistency and accuracy by the parameters like mean and percentages. Chi- square test and Fischer Exact test are used. The differences of above parameters were tested by the parametric tests like 'Z' and 't' and non-parametric like x^2 tests test, which was applicable wherever. The results of ELISA and IIFT were compared by Mcnemar's x^2 test and confirmed by 'Z' test of proportions. The above statistical procedures were performed by IBM SPSS Statistics 20. The P-Values of less than 0.05 were considered as statistically significant ($P<0.05$).

5.3 Result Analysis

The selected 50 study subjects were analysed based on age and sex, the results of the analysis are tabulated in table 1.

Table 1.Age and sex wise distribution in study group

Age (in years)	**Male**		**Female**		**Total**	
	No.	**%**	**No.**	**%**	**No.**	**%**
< 40	0	0	1	11.1%	1	2%
41-50	4	9.8%	3	33.3%	7	14%
51-60	18	43.9%	2	22.2%	20	40%
61-70	16	39%	3	33.4%	19	38%
>70	3	7.3%	0	0	3	6%
Total	**41**	**100%**	**9**	**100%**	**50**	**100%**

Of the 50 patients, 41 were males. Of this, 4 (9.8%) were in the age group of 41-50 years and 18 (43.9%) were in the age group of 51-60 years and 16 (39%), 3(7.3%) were in the age group of 61-70 years and > 70 years.

Out of 50 patients, 9 were females. Of this one (11.1%) was in the age group of < 40 years and 3(33.3%) were in the age group of 41-50 years, 2 (22.2%) were in the age group of 51-60 years,3 (33.4%) were in the age group of 61-70 years and none above 70 years.

FIGURE:1

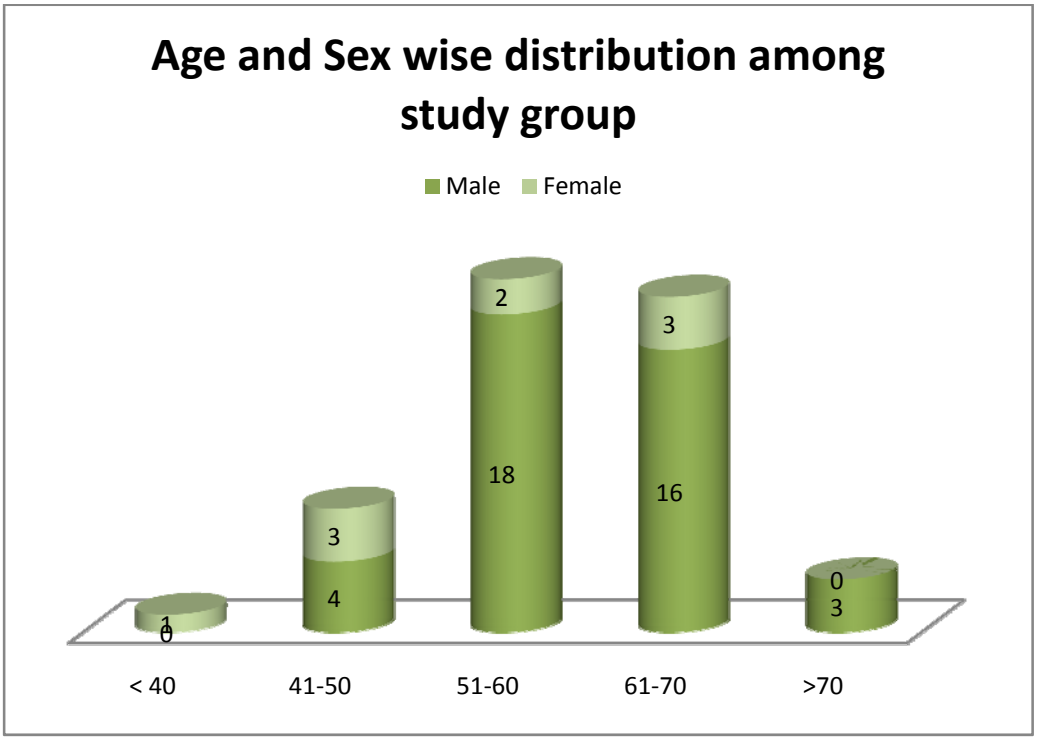
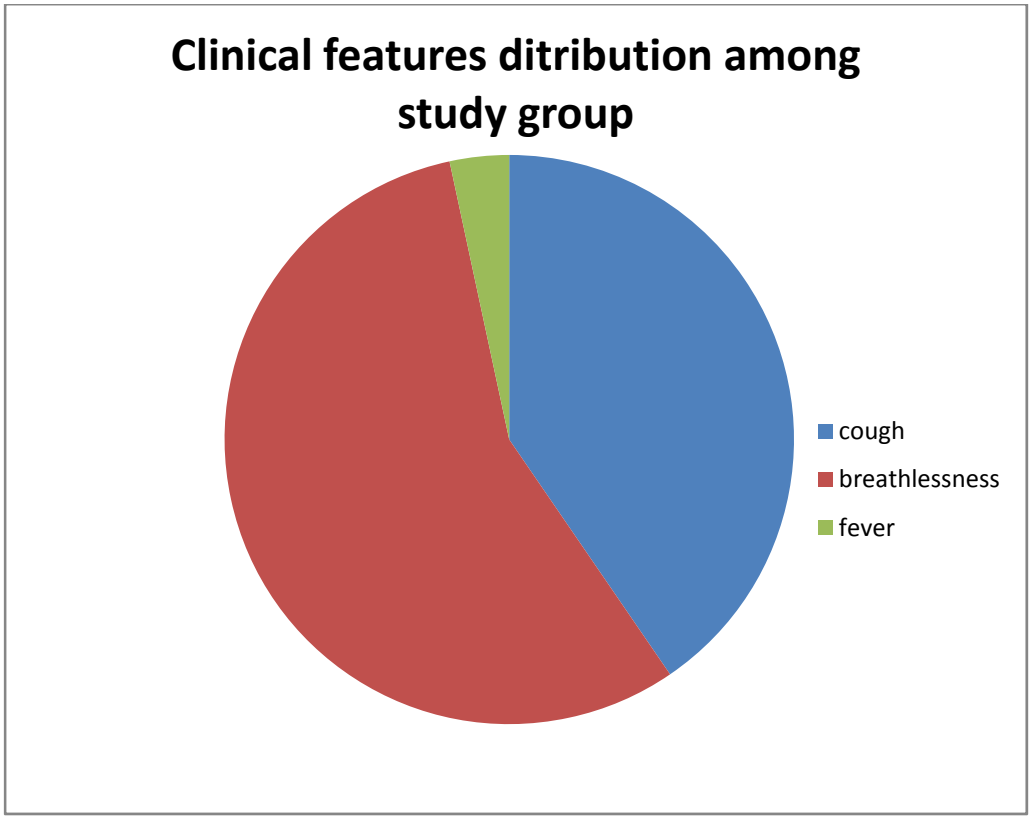

Age and Sex wise distribution among study group
Male
Female
1
0
3
4
2
18
3
16
0
3
< 40
41-50
51-60
61-70
>70

Table 2. Distribution of associated clinical features in study group.

Clinical features	No. of patients (n =50)	Percentage
Cough	36	72%
breathlessness	50	100%
fever	3	6%

This table shows (table 2) that among the 50 cases studied, 36 (72%) were associated with cough, 50 (100%) had breathlessness, 3 (6%) were presented with fever.

FIGURE : 2



The above figure shows the clinical features, cough, breathlessness, fever among study group.

Table 3: Smoking history among study group

Smoking history	No of patient	percentage
Smoker	35	70%
Non smoker	15	30%
total	50	100%

The above table shows smoking history distribution among the 50 subjects. Out of this ,35 (70%) were smokers and 15 (30%) were non smoker

FIGURE : 3

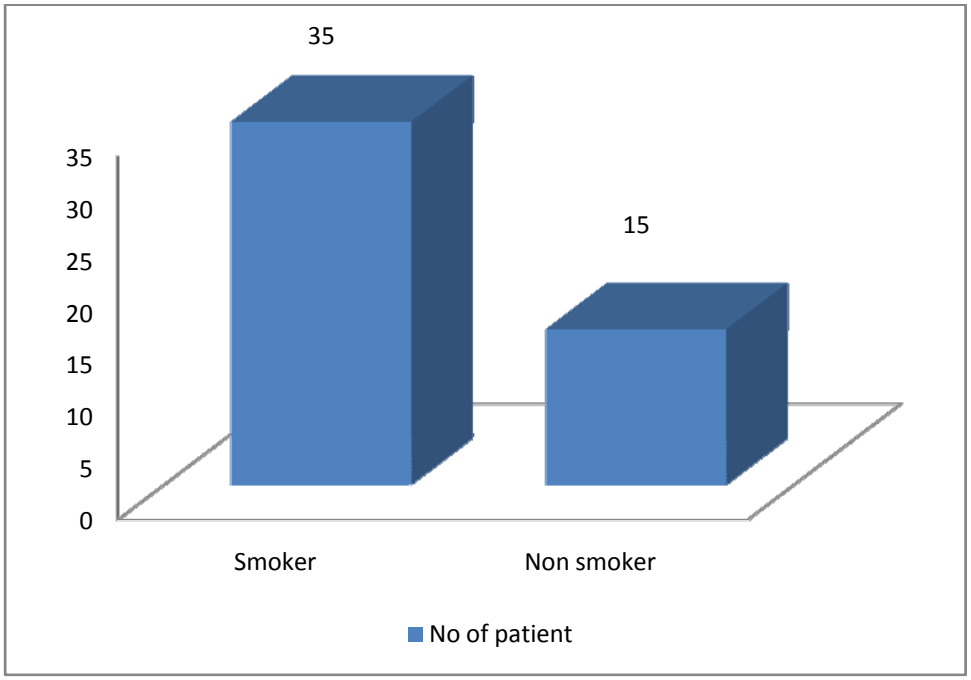


Table :4 – frequency of risk factors among study group

Risk factor	Present	Absent
DM	3	47
HTN	6	44
ALCOHOLISM	27	23

The above table shows among the 50 subjects, 3 were DM and 47 were Non DM, 6 were HTN and 44 were Non HTN and 27 we re Alcoholic and 23 were Non alcoholic

FIGURE: 4

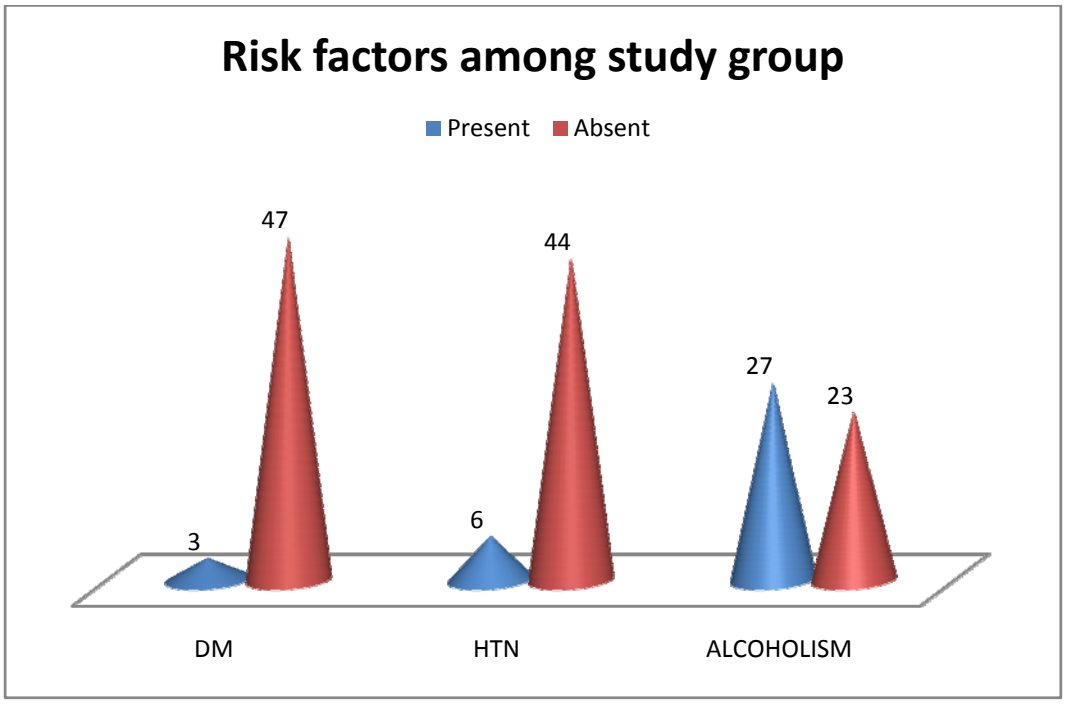
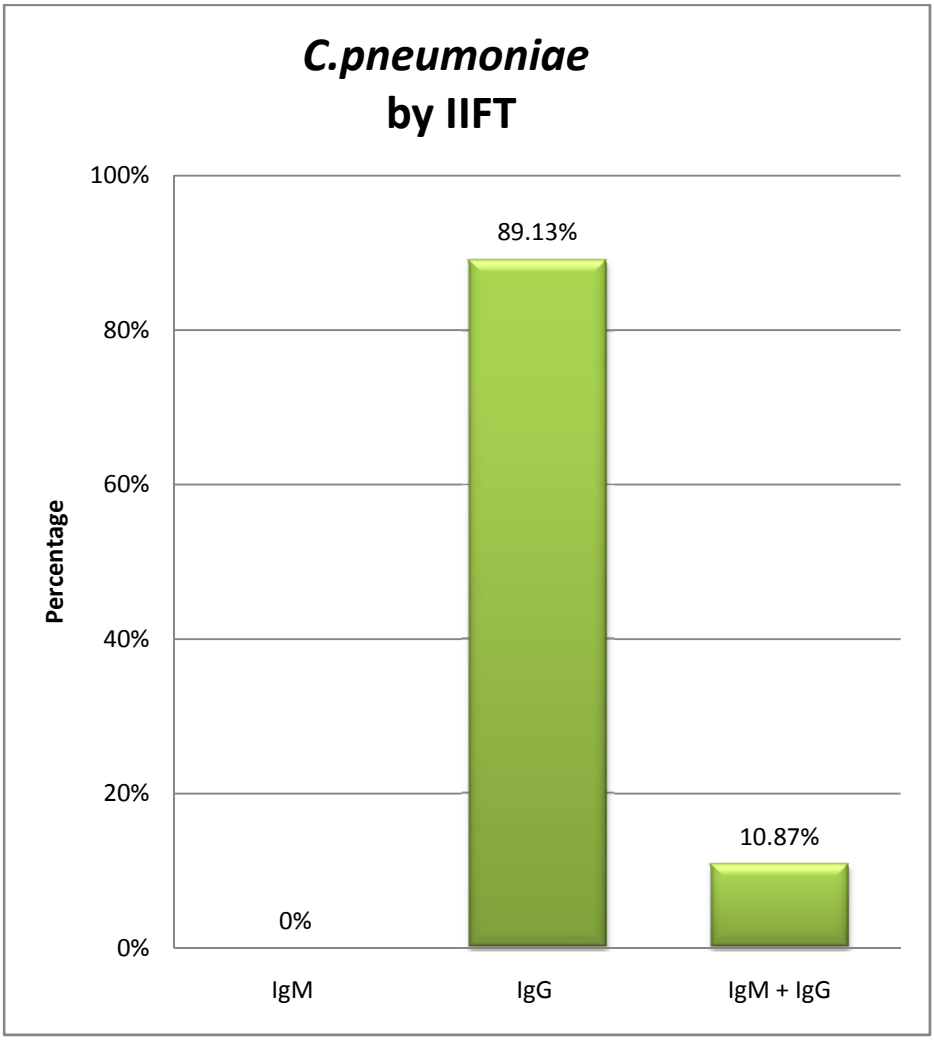


Table 5: Detection of C. *pneumoniae* antibodies by IIFT

Parameters	No of positives	Percentage
IgM alone	0	0%
IgG alone	41	89.13%
Both IgM + IgG	5	10.87%
Total	46	100%

The above table shows that among 46 IIFT positive cases, no one showed positive for IgM only, 41 for IgG only,5 for IgM+ IgG.

FIGURE : 5



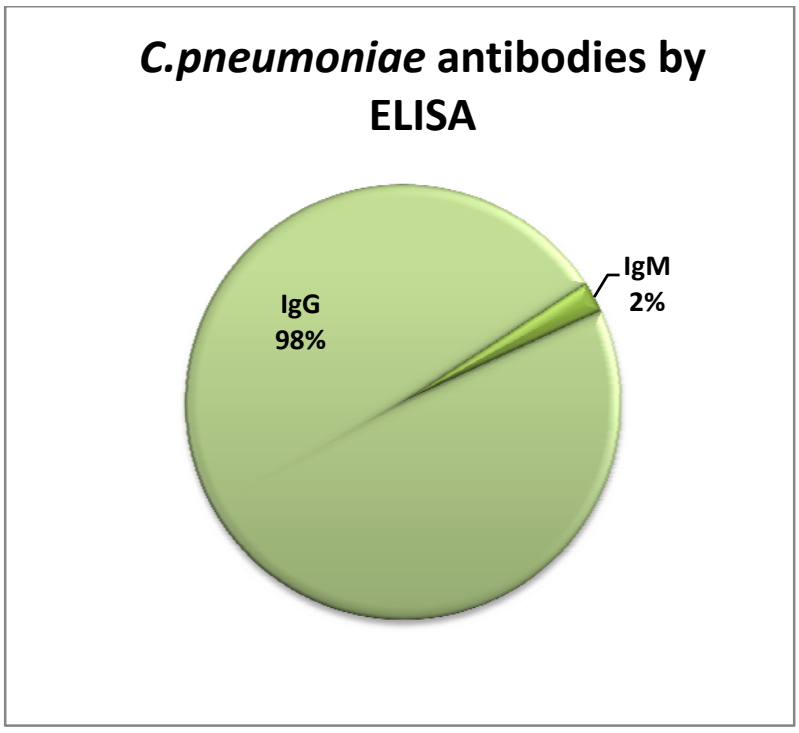
The above figure shows 89.13% for IgG and 10.87% for both IgM+IgG.

Table 6. Detection of *C.pneumoniae* antibodies by ELISA

Parameters	No of positives	Percentage
IgM alone	0	0%
IgG alone	42	97.67%
Both IgM+IgG	1	2.33%
Total	43	100%

The above table shows among 43 ELISA positive cases , no positives for IgM only, 42 were IgG only and only one was positive for both IgM+ IgG.

FIGURE: 6



The above figure shows 98% was IgG and remaining 2% were IgM positives.

Table 7. Evaluation of *C.pneumoniae* IgM antibody detection by ELISA against IIFT

ELISA	IIFT		Total
	Positive	Negative	
Positive	1	0	1
Negative	4	45	49
Total	5	45	50

Among the 50 samples tested, 5 were positive for *C.pneumoniae* IgM antibody Detection of *Chlamydia pneumoniae* IgM antibody by ELISA was evaluated for its sensitivity and specificity against IIFT.

Sensitivity = $\frac{TP}{TP+FN} = \frac{1}{5} \times 100 = 20\%$

Specificity = $\frac{TN}{TN+FP} = \frac{45}{45} \times 100 = 100\%$

Positive predictive value (PPV)= $\frac{TP}{TP+FP} = \frac{1}{1} \times 100 = 100\%$

Negative predictive value (NPV) = $\frac{TN}{TN+FN} = \frac{45}{49} \times 100 = 92\%$.

From the above table, sensitivity of ELISA test was found to be 20% when evaluated against IgM IIFT, as a reference test. Specificity was 100% when compared to IIFT and positive and negative predictive values were 100% and 92% respectively. According to the Mc Nemer test the p value is > 0.05 which is not significant suggests that the ELISA is not more sensitive than the gold standard test IIFT.

Table 8: Evaluation of C. *pneumoniae* IgG antibody detection by ELISA against IIFT.

ELISA	IIFT		Total
	Positive	Negative	
Positive	42	2	44
Negative	4	2	6
Total	46	4	50

Among the 50 samples tested 46 were IgG antibody positive.

Detection of *C.pneumoniae* IgG antibody by ELISA test was evaluated for its sensitivity and specificity against IIFT as a reference test.

Sensitivity = $\frac{TP}{TP+FN} = \frac{42}{46} \times 100 = 91\%$

Specificity = $\frac{TN}{TN+FP} = \frac{2}{4} \times 100 = 50\%$

Positive predictive value = $\frac{TP}{TP+FP} = \frac{42}{44} \times 100 = 91\%$

Negative predictive value = $\frac{TN}{TN+FN} = \frac{2}{6} \times 100 = 33\%$.

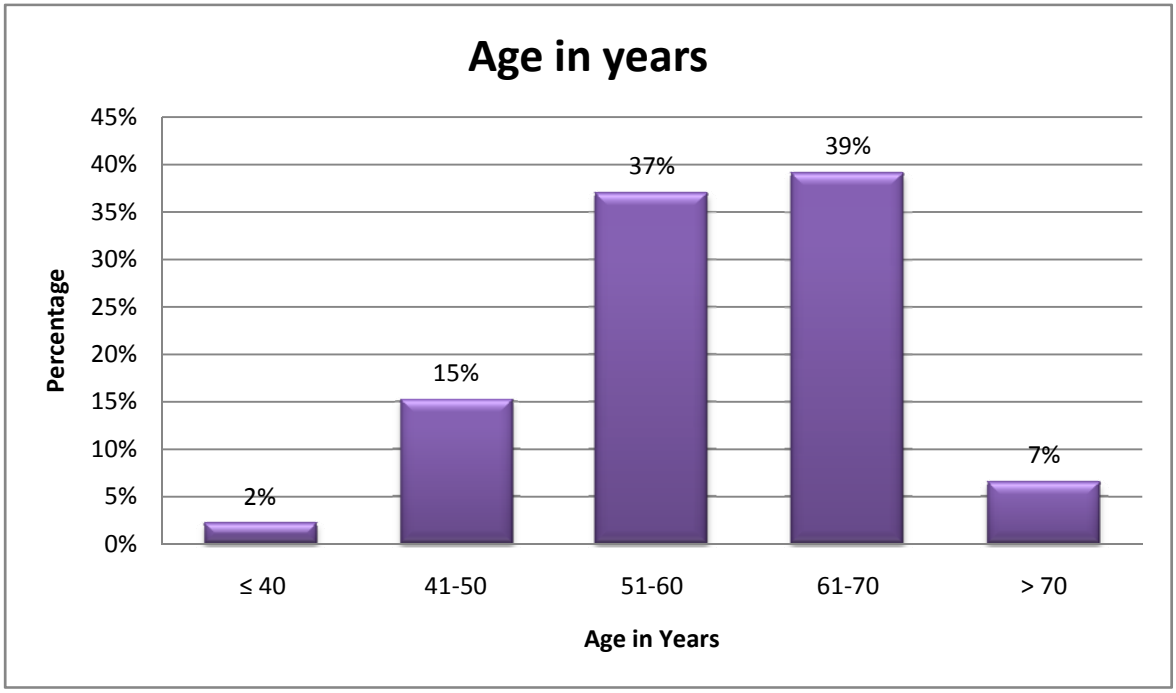
From the above table, the sensitivity of ELISA was 91% when evaluated against IgG for IIFT, as a reference test. Specificity was 50% compared to IIFT and positive and negative predictive values were 91% and 33% respectively.

TABLE 9. Age wise distribution among *C. pneumoniae* positive cases by IIFT

Age in years	Positive cases	Percentage
≤ 40	1	2%
41-50	7	15%
51-60	17	37%
61-70	18	39%
> 70	3	7%
Total	46	100%

The above table shows that majority of positive cases in the age group of61-70years.out of 46 positive cases shown by IIFT, only one subject was below 40 years. Among 51-60 years 17 were positives by IIFT.

FIGURE: 7



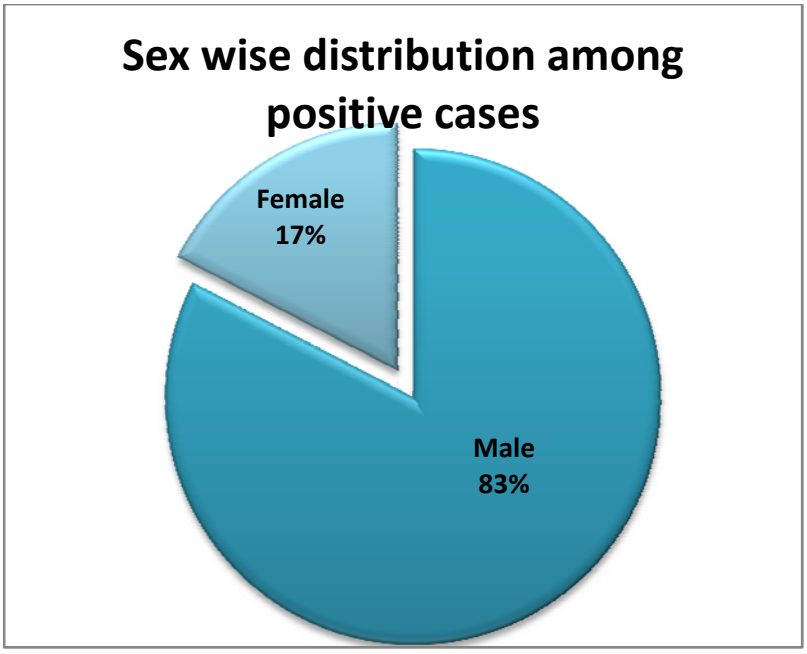
The above figure shows, among the 46 positive cases by IIFT 37% were in the age group of 51 – 60 years and 39% were in the age group of 61 – 70 years.

TABLE :10- Sex wise distribution among *C. pneumoniae* positive cases by IIFT

Sex	Positive cases	Percentage
Male	38	82.61%
Female	8	17.39%
Total	46	100%

Sex wise distribution of Chlamydia pneumonia positive cases in the above table shows that males are more affected than the females. Out of 46 positive cases detected by IIFT 38 were males and 8 were females. The ratio of male to female was found to be 4.8:1

FIGURE:8



The figure shows that out of 46 positive cases detected by IIIF 83% were males and 17% were females.

Table 11- Association of smoking among *C. pneumoniae* positive cases by IIFT

Risk factor	Positive	Negative
Smokers (n=35)	33	2
Non smokers (n=15)	13	2

Association of smoking *in Chlamydia pneumoniae* positive cases is shown in the table. Out of 46 positive cases detected by IIFT 33 cases had H/O smoking whereas 13 were Non smokers. According to fisher´s exact test the p value was 0.574. It was found to be statistically non significant.

FIGURE: 9

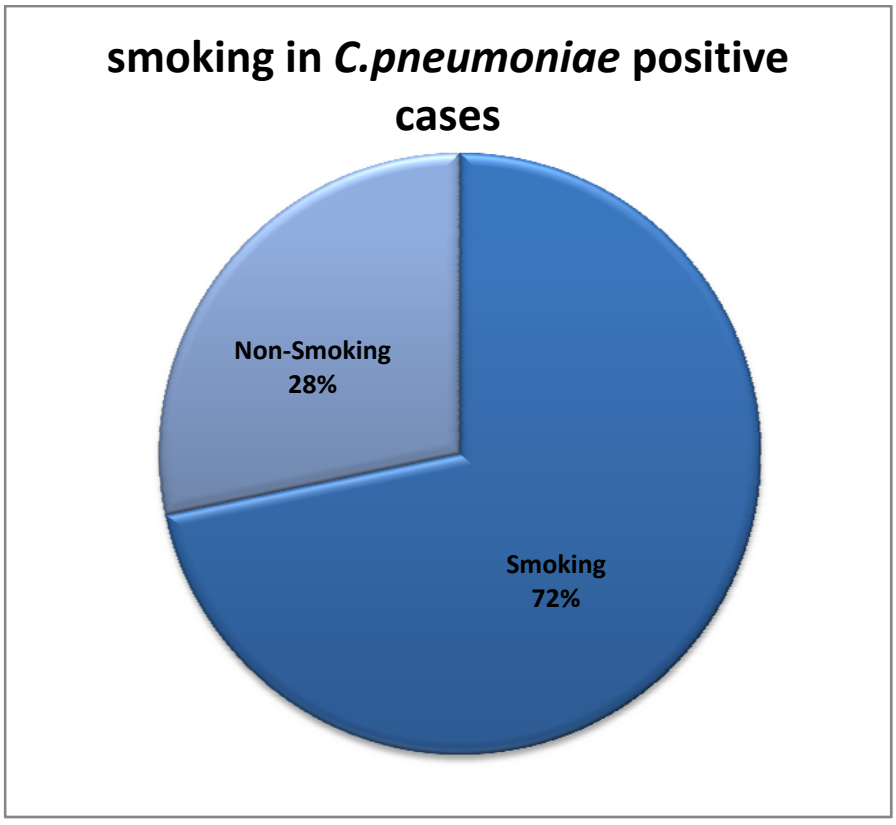


Table 12: Association of cough in *C. pneumoniae* positive cases by IIFT

Cough	Positive	Negative
Present (n=36)	32	4
Absent (n=14)	14	0

The above table shows that out of 46 positive cases, 32 patients were presented with cough and remaining 14 patients did not have complaining of cough. According to fishers exact test the p value was 0.566. it was found to be statistically non significant.

FIGURE: 10

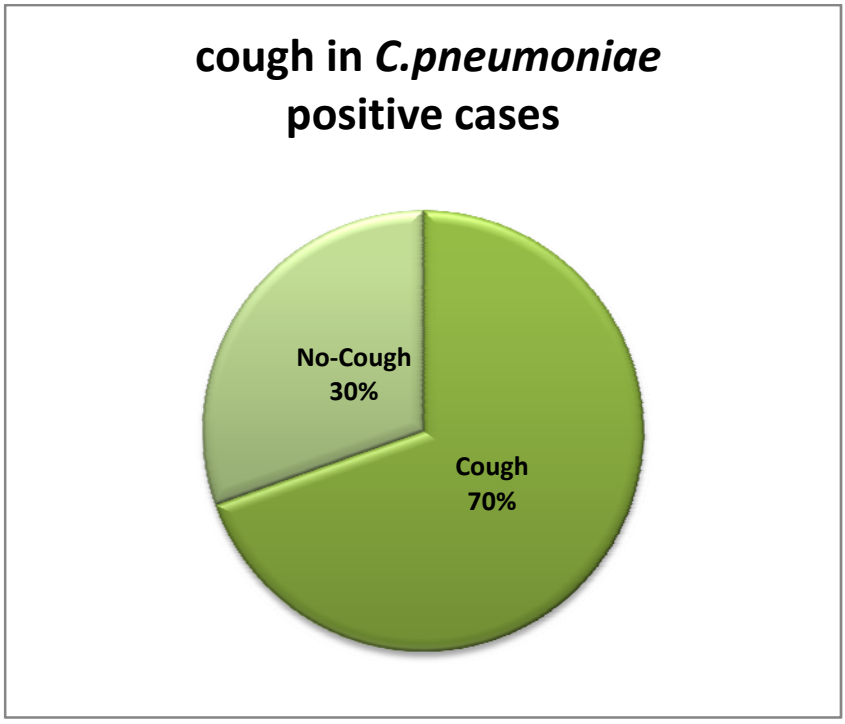


Table 13: Association of dyspnoea in *C. pneumoniae* positive cases by IIFT

	Dyspnoea	Positive	Negative
Dyspnoea	Present (n=50)	46	4
	Absent (n=0)	0	0

The above table shows that among 46 positive cases, all the 46 patients had difficulty in breathing.

Table 14: Association of fever in *C. pneumoniae* positive cases by IIFT

	Fever	Positive	Negative
Fever	Present (n=3)	3	0
	Absent (n=47)	43	4

The above table shows that, out of 46 positive cases, 3 had fever and remaining 43 didn´t have fever. According to fisher´s exact test the p value was 1. It was found to be statistically non significant.

FIGURE:11

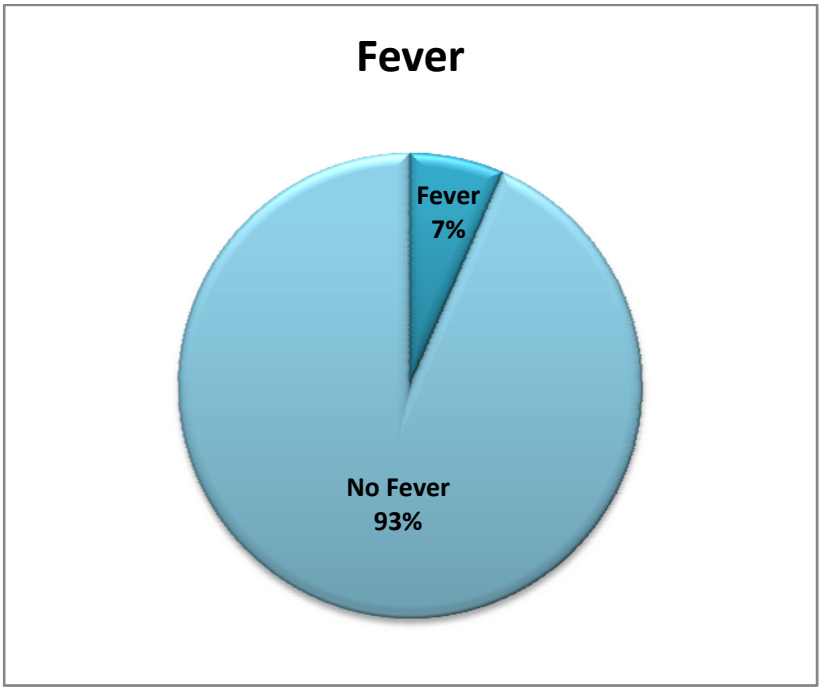
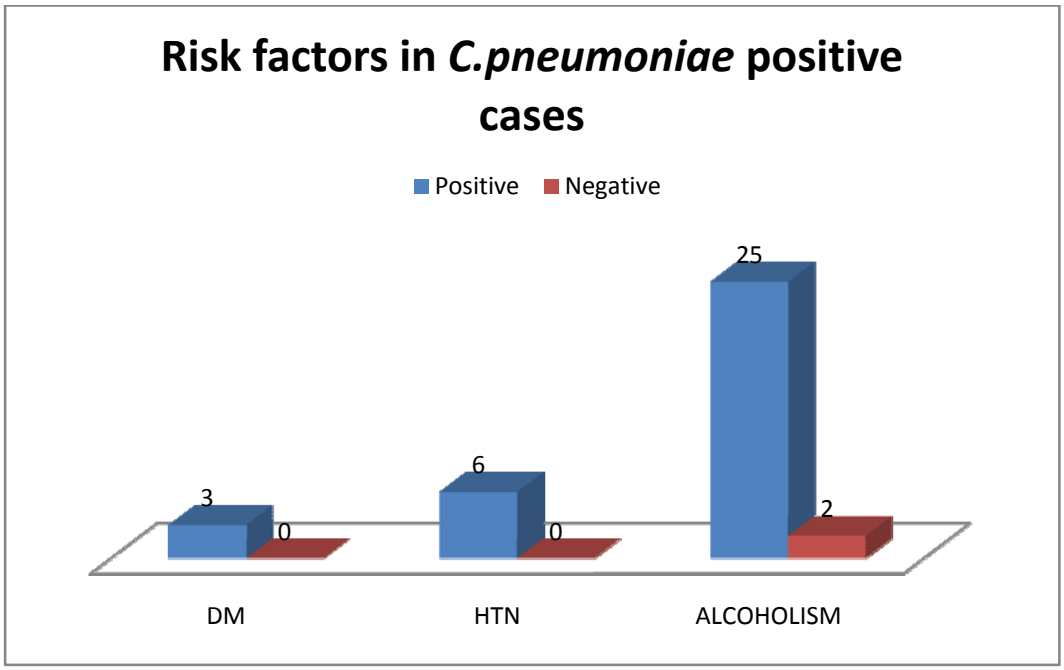


Table : 15- Association of risk factors in *C. pneumoniae* positive cases by IIFT

Risk factor	Positive	Negative
DM	3	0
HTN	6	0
ALCOHOLISM	25	2

Table 15 shows, among 46 positive cases 3 had DM, 6 had HTN and 25 were Alcoholic.

FIGURE : 12



The above figure shows distribution of risk factors, like DM, HTN and Alcoholic among the 46 positive cases by IIFT.

Discussion

6. DISCUSSION

Chlamydia pneumoniae is a respiratory pathogen, and most people are infected by the age of 10 years[70]. Many studies have shown that the antibody prevalence rises with age in adult populations. *C. pneumoniae* causes upper and lower respiratory tract infections in humans and causes about 10% of the pneumonia cases in adults worldwide[5]. Persistent *C. pneumoniae* infections have been associated with several chronic diseases, such as asthma and chronic obstructive pulmonary disease. After an acute infection, IgM titers usually fall within 2 months and normalize within 4 to 6 months. Elevated IgG levels may persist for several years and occasionally be detectable over 3 years after the acute infection, elevated IgG antibodies may also indicate on going chronic infection.

In this study, examined the possible role of *C. pneumoniae* in episodes of acute exacerbation of COPD. In the present study it was demonstrated that approximately 10.9% of the COPD patients had serological evidence of acute *C. pneumoniae* infection at the time of exacerbation by using the combination of the IIFT test and ELISA and that chronic *C. pneumoniae* infection was also common**.** Since direct isolation of *C.pneumoniae* is difficult, serology plays an important role in confirming *C.pneumoniae* infection. The IIFT test is generally accepted as the gold standard for the serological diagnosis of *C.pneumoniae* infection. However, interpretation of specific and non-specific fluorescence patterns requires experience and skill, and the interpretation is very difficult and subjective. In addition, cross-reactions between *C. pneumoniae* and other

Chlamydial species have been reported. The result of the study showed that *C. pneumoniae* IgG antibody is suggestive of persistent infection was significantly more in elderly with COPD patients. Several studies in adults have shown that the association between the presence of *C.pneumoniae* IgG antibody seropositivity and chronic persistent asthma[80,81]. The possible mechanisms to increase the severity of COPD may be the result of increased production of inflammatory cytokines.

6.1 Age and sex wise distribution in study group

The present study shows that out of 50 suspected cases, males are found to be predominant than the females in the ratio of 4.6:1.

Male preponderance was also observed in similar studies conducted by Sonata *et al*[73]. Smoking is observed more among males than females and it is a known risk factor for development of COPD. Majority of the suspected COPD cases in the study group belong to the age group of 51-70 years in the present study. This result is similar to the study conducted by Allegra *et al*[71].

The prevalence of *C.pneumoniae* infection depends on the subject's age, geographic area of residence and the prevalence of other chronic diseases. Endemicity of *C.pneumoniae* infection is more in Asia than in Western countries[3].

6.2 Distribution of associated clinical features, laboratory findings in study group:

The present study shows that among the 50 *C.pneumoniae* suspected cases, 100% was associated with dyspnoea, and 72% with cough and 6% had fever. 70% were smokers, 54% were alcoholism, 6% were DM, 12% were HTN, and 46% were associated with X-ray findings.

This was similar to the study conducted by Georgios *et al*[74] and in contrast to a study by Sanjay *et al* [70]. Georgios *et al*[74], study showed among the 75 COPD patients, all were associated with dyspnoea, 55% were smokers, 84% with cough. In David *et al*[82], study revealed that among 147 patients 87% with dyspnoea, 44% were smokers and 11% had fever.

6.3 Detection of *C.pneumoniae* antibodies by IIFT:

In the present study among the 50 patients tested, 46 were found to be *C.pneumoniae* antibody positive by IIFT. Among the 46 positive cases, none were positive for IgM antibodies alone, whereas 41 cases were positive for IgG and 5 were positive for both IgM and IgG antibodies. Cells infected with *C.pneumoniae* are used as antigen for IIFT.

In his study, Kwang-Jun Lee *et al*[77] among 19 patients in acute phase, 11(58%) were positive for IgM and 5(26%) were positive for IgG. However Sanjay *et al*[70], studied among 54 patients, 26 were positive for IgG and no positive cases for IgM.

6.4 Detection *of C.pneumoniae* antibodies by ELISA:

The present study shows 43 ELISA positive cases among the 50 cases tested. among them, 42 (97.67%) were positive for IgG antibodies only, one (2.33%) for IgM+IgG and there were no IgM alone positives cases.

In a similar study conducted by Verkooyen *et al*[9] among showed that out of 125 ELISA positive cases, all the 125 were positive for IgG antibody only. None of the cases were positive for IgM antibody. Agarwal A, Chander Y[79] studied

among 60 patients for *C.pneumoniae* ELISA, all the 60 were positive for IgG and no positive cases for IgM.

6.5 Comparison of positivity by ELISA and IIFT in detection of *C.pneumoniae* infection:

The positivity rate of ELISA was 86% and it was 92% by IIFT. In the present study *C.pneumoniae* infection was found in 92 % of adult patients with COPD.

In a Norethern india study done by Sanjay *et al*[70], observed that the prevalence of *C.pneumoniae* was 40.62%. And there was a high prevalence of *C.pneumoniae* 85.5% in elderly group conducted by Sontana *et al* [73].

6.6 Evaluation of ELISA against IIFT as gold standard:

The *Chlamydia* ELISA used in this study is based on *chlamydial* LPS and MOMP antigens intended for the detection of serum anti-LPS IgG, and IgM antibodies in patients with *chlamydial* infections. The advantages of these tests over IIFT and culture include reproducibility, objective analysis, and short performance time. However, this test cannot differentiate between *chlamydial* species due to the genus-specific nature of *chlamydial* LPS**.**

The sensitivity of *C.pneumoniae* IgM ELISA was 20% when evaluated against IIFT, as a reference test. Specificity of ELISA was 100% compared to IIFT and positive and negative predictive values were 100% and 92% respectively. The sensitivity of *C.pneumoniae* IgG antibody ELISA was 91% while the specificity was 50% when evaluated against IIFT.

The sensitivity calculated from this study is in contrast to the study conducted by Gutierrez *et al* at 2001. Similar low sensitivity and high specificity for ELISA was reported by Kenneth Person *et al*[83] at 2000 with 36% sensitivity and 95% specificity. Hence all the samples negative by ELISA have to be confirmed by a more sensitive test like MIF/IIFT or PCR.

6.7 Evaluation of *C.pneumoniae* IgM antibody by ELISA and IIFT:

IgM antibody appears 2-3 weeks after the onset of illness and generally, after 2-6 months the IgM will not be detectable.

IgM antibody was detected in 5 cases by IIFT and in one case by ELISA. All these patients also had coexistent IgG antibodies. In a similar study conducted by Verkooyen *et al*[9] among COPD patients out of 125 ELISA positive cases, none of the cases were positive for IgM antibody.

Kwang-Jun Lee *et al*[77] observed that 48% of the study population was positive for IgM antibodies at the acute phase of respiratory tract infections in Korea, and 16% were positive during the convalescent phase. Sontana *et al*[73] revealed that only 4% had IgM in their serum during exacerbation of COPD.

The difference in sensitivity and specificity of IgM based assays may be because they are strongly influenced by the quality of the antigen used and vary greatly between commercially available products.

6.8 Evaluation of *C.pneumoniae* IgG antibody by ELISA and IIFT:

A delayed IgG response appears 6-8 weeks after the illness. IgG antibodies can indicate either chronic or repeated infections. *C.pneumoniae* IgG antibodies were detected in 46 patients by IIFT and 43 patients by ELISA.

In a Similar study Sontana *et* al[73] revealed high prevalence of *C.pneumoniae* IgG (75.8%) among the study group. A study done in Madyapradesh by Agarwal *et al*[79] in 2008 revealed that *C.pneumoniae* IgG antibody positivity rate in patients with bronchial asthma was significantly high (80%). A high prevalence of IgG was reported in a study population by Seattle, in the United States of America (73%) .

IgG antibodies have been shown to persist for long periods and decline very slowly. This higher prevalence may be due to bronchial hyper responsiveness to the *C.pneumoniae* antigen. High levels of IgG antibodies are of diagnostic value in chronic *chlamydial* infection.

6.9 Age wise distribution among positive cases:

The present study shows that majority of positive cases are in the age group of 61-70 years. Out of 46 positive cases shown by IIFT, 35(76%) were in the age group of elderly peoples.

Agarwal *et al*[79] (80%) and Sontana *et al*[73] (86.4%) also reported the similar results in which elderly age groups forms the majority of positive cases. However, Sanjay *et al*[70] revealed that seroprevalence of *C.pneumoniae* is higher in asthmatic children in the age group of 5-14 years conducted at PGIMER, Chandigarh at 2002.

This high prevalence in elderly peoples in this study may be due to repeated infections of *C.pneumoniae,* risk factors like DM, decreased immunity and it leads to persistence of infections.

6.10 Sex wise distribution among positive cases:

Sex wise distribution of *C.pneumoniae* positive cases in the present study shows that males are more affected than the females. Out of 46 positive cases detected by IIFT, 38(82.6%) were males and 8 (17.39%) were females in the ratio of 4.8:1.

Sontana *et al*[73] also showed similar results where males are more predominant than the females in the ratio of 17:1. Seropositivity rate was higher in males as revealed by Grayston *et al* [69].

High prevalence among males is probably due to more outdoor activities by males in comparison to females which results in more exposure to infection.

6.11 Association of smoking in positive cases:

Smoking is a main predisposing factor in COPD patients causing *C.pneumoniae* infection. In the present study out of 46 positive cases 33 (71.7%) were smokers and 13(28.3%) were non smokers. Cigarette smoking had the higher rate of infection with *C.pneumoniae*. Smoking is well known important risk factor for COPD patients through alteration in mechanisms of the host defense system. In the present study, there was association found between smoking and *C.pneumoniae* infection.

In contrast Ruiz *et al* study in 1999 which was reported that there was no association between the cigarette smoking and infection due to atypical bacterial pathogens especially *C.pneumoniae*.

Sontana *et al*[73] (11) studied among 127 COPD, 97 patients had H/O of smoking and the seroprevalence of *C.pneumoniae* was detected as 96.1%. This

prevalence was higher among smokers when compared with non smokers. In contrast Naoyuki *et al*[76] studied among 366 COPD adults 240 were smokers and showed 24% prevalence.

6.12 Association of cough in positive cases:

In the present study out of 46 positive cases, 32 (69.5%) presented with cough and 14 (30.43%) did not present with cough.

C.pneumoniae is a well-known cause of persistent cough in both children and adults. Naoyuki *et al* [76] showed that about 20% of patients presented with cough lasting for more than 2 weeks had serological evidence of acute *C.pneumoniae* infection. It may be accountable for a substantial proportion of prolonged coughing illnesses. Positivity rate by IIFT was 38% among children with persistent cough.

In contrast Birkeback *et al* in 2000 detected only 3 out of 201 adult patients with chronic coughs by *C.pneumoniae*. Kwang-Jun Lee *et al*[77] studied *C.pneumoniae* prevalence among 137 students and demonstrated that72 (58.3%) of them had cough.

6.13 Association of dysponea in positive cases:

In the present study, out of 46 positive cases, all patients had difficulty in breathing.

In a similar study by Georgios *et al*[74] among the 75 COPD patients, 93% of patients who showed difficulty in breathing had serological evidence for *C.pneumoniae* infection.

6.14 Association of fever in positive cases:

The present study showed, among the positive cases only 3(6.5%) had fever.

A similar study by Sontana *et al*[73] showed that among 127 elderly COPD patients, the seropositivity rate was 96.1% and only 3.9% positive cases presented with fever. David *et al*[82] studied seroprevalence of CP infection among 147 adult men, among this 11% of positive cases presented with fever.

The present study shows an increase in positivity when a single serum from the patient was subjected to a combination of ELISA and IIFT rather than subjecting to ELISA.

Thus in this study an increase in detection of *C.pneumoniae* infection when both IgG and IgM antibodies were tested. ELISA was found to be less sensitive than IIFT. IgG antibodies were more prevalent than IgM antibodies and also found to be high among the elderly age group. This could indicate a chronic persistent *C.pneumoniae* infection among the tested COPD patients.

Summary

7. SUMMARY

- In the present study 46 cases were positive for *C.pneumoniae* by IIFT.
- In the present study 43 cases were positive for *C.pneumoniae* by ELISA.
- Sensitivity, specificity, PPV, NPV for IgM antibody was 20%, 100%, 100% and 92% respectively. The sensitivity, specificity, PPV, NPV for IgG antibody was 91%, 50%, 91% and 33% respectively.
- In the present study *C.pneumoniae* infections were more common in the age group of 51-70 years.
- *C.pneumoniae* infections more in males (83%) when compared to females (17%) with a male: female ratio of 4.8:1.
- The *C.pneumoniae* infection rate was highest in patients presented with H/O smoking (72%).
- In the present study majority of *C.pneumoniae* infections were common with patients having coughs more than 2 weeks (70%).
- In the present study *C.pneumoniae* infections were more common among the patients with severe grade of dyspnoea (92%).
- Fever was a less significant symptom among the *C.pneumoniae* infections (6.5%).

Conclusion

8. CONCLUSION

- Our preliminary data represent the first evidence of association between *C.pneumoniae* infection and COPD in our area.
- This study highlights the high prevalence of *C.pneumoniae* infections among adult COPD patients.
- *C.pneumoniae* infection was found to be particularly high in the elderly groups.
- This suggests that it plays a substantial role in causing acute or chronic persistent infections in COPD patients.
- ELISA was found to be less sensitive than IIFT, but with the cost constrains, ELISA test is very simple, easy and cost effective that can be easily adopted in the clinical laboratory.
- These *C.pneumoniae* infections may make a significant contribution to morbidity in COPD patients. Further studies are needed to define more effective treatment strategies.

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Annexure

SEROPREVALENCE OF *CHLAMYDIA PNEUMONIAE* INFECTIONS AMONG ADULT WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASES BY ELISA AND IMMUNOFLUORESCENCE

PROFORMA

Name :

Age / Sex :

Occupation :

Address :

Clinical Diagnosis :

Present Complaints

H/O Fever & Duration :

H/O Cold :

H/O. Cough & Duration :

H/O Difficulty in Breathing :

Past History :

General Examination

DM		Non DM	
HT		Non HT	
Alcoholic		Non Alcoholic	
Smoker		Non Smoker	

CVS :

RS :

CNS :

Investigation :

TC	DC	ESR	Hb

X-Ray Findings :

Results ELISA IgM : IgG :

IMMUNOFLUORESCENCE IgM : IgG :

MASTER CHART

S.No	Age	Sex	Occupation	Cough with expectoration	Duration	Brelessness	DURATION	Grade	Fever	H/OSmoking	H/OAlcohol	H/O hypertension	H/O Diabetes mellitus	X - ray findings	Total Count	Differential Count	ELISA IgM	OD value	ELISA IgG	OD value	IIFT IgM	IIFT IgG
1	60	MALE	Coolie	P	2 weeks	P		III	N	N	N	P	N	P	P	P	N	0.074	P	0.9112	N	P
2	81	MALE	Agricutural worker	P	1	P		III	P	N	N	N	N	N	N	N	N	0.0271	P	0.8622	N	P
3	63	MALE	Coolie	P	1	P	1	II	N	P	N	N	N	P	N	N	N	0.0265	P	1.175	N	P
4	65	MALE	Coolie	P	12	P	12	II	N	P	P	N	N	P	P	P	N	0.0556	P	0.9242	N	P
5	65	MALE	Coolie	P	5 D	P	5D	II	N	P	P	P	P	P	N	N	N	0.1244	P	1.0468	P	P
6	74	MALE	Unemployee	P	2	P	2	III	N	P	P	P	P	P	N	N	N	0.2054	P	0.3297	N	P
7	65	MALE	Coolie	P	1	P	1	II	N	P	P	N	N	N	N	N	N	0.0392	P	0.8785	N	P
8	63	MALE	Coolie	N		P	5D	III	N	P	P	N	N	P	N	N	N	0.0607	P	0.4909	N	P
9	67	MALE	Coolie	P	4 W	P	7D	II	N	P	P	N	N	N	N	N	N	0.0432	P	0.9994	P	P
10	60	MALE	Coolie	P	4W	P	4W	III	N	P	N	N	N	N	N	N	N	0.0589	P	1.3554	N	P
11	60	MALE	Coolie	P	10D	P			P	N	N	N	N	N	N	N	N	0.0505	P	1.2607	N	P
12	69	MALE	Coolie	P	1 W	P	1W	III	P	P	P	N	N	P	N	N	N	0.05	P	0.9311	N	P
13	65	MALE	Coolie	P	2W	P	2W	II	N	P	P	N	N	N	P	P	P	0.5666	P	0.8115	P	P
14	45	MALE	Coolie	P	4W	P	2W	II	N	P	P	N	N	P	N	N	N	0.025	P	0.5338	N	P
15	60	FEMALE	House wife	P	1W	P	1W	II	N	N	N	N	N	P	N	N	N	0.1885	N	0.2352	N	N
16	60	MALE	Farmer	P	1W	P	1W	III	N	P	N	N	N	N	N	N	N	0.1637	P	1.0001	N	P
17	63	MALE	Coolie	P	3W	P	8W	III	N	P	N	N	N	N	N	N	N	0.0905	P	0.966	N	P
18	59	MALE	Coolie	P	24W	P	24W	III	N	P	P	N	N	P	N	N	N	0.0799	P	1.2649	N	P
19	60	MALE	Farmer	N		P	2 Y	III	N	P	P	N	N	N	N	N	N	0.111	N	0.2906	N	P
20	60	MALE	Coolie	P	1W	P	1W	III	N	P	P	N	N	N	N	N	N	0.0444	P	1.1256	N	P
21	59	MALE	Coolie	P	10D	P	10D	II	N	N	N	N	N	N	N	N	N	0.1689	P	0.8353	N	P
22	69	MALE	Farmer	N		P	7 Y	III	N	P	P	N	N	P	N	N	N	0.025	P	1.2745	N	P
23	50	MALE	Mill worker	P	2W	P	10Y	III	N	P	N	N	N	P	N	N	N	0.0559	P	0.9616	N	P
24	54	MALE	Farmer	N		P	6Y	III	N	P	P	N	N	P	N	N	N	0.0649	P	1.1738	N	P

25	65	FEMALE	Home Maker	P	3W	P	3W	III	N	N	N	N	N	N	N	N	N	0.0632	P	0.747	N	P
26	56	MALE	Coolie	N		P	8Y	III	N	N	N	N	N	N	N	N	N	0.061	P	1.0636	P	P
27	67	MALE	Farmer	N		P	5Y	III	N	P	N	N	N	P	N	N	N	0.0707	P	0.9026	N	P
28	51	FEMALE	Home Maker	P	2W	P	3Y	III	N	N	N	N	P	P	N	N	N	0.0472	P	0.7497	N	P
29	54	MALE	Watchman	N		P	28W	III	N	P	P	N	N	P	N	N	N	0.0368	P	0.9305	N	P
30	42	FEMALE	Home Maker	N		P	7Y	III	N	N	N	N	N	N	N	N	N	0.1066	P	0.6541	N	P
31	60	MALE	Farmer	P	2W	P	13Y	III	N	P	P	P	N	N	N	N	N	0.0334	P	0.499	N	P
32	50	MALE	Farmer	P	10D	P	6M	II	N	P	P	N	N	N	N	N	N	0.032	P	1.0704	N	P
33	60	MALE	Coolie	P	1M	P	10Y	III	N	P	P	N	N	N	N	N	N	0.0149	P	0.622	N	P
34	47	FEMALE	Coolie	P	1W	P	1Y	III	N	N	N	N	N	N	N	N	N	0.0257	P	0.905	N	P
35	65	MALE	Coolie	N		P	3Y	III	N	P	P	N	N	P	N	N	N	0.2074	P	1.0196	N	P
36	54	MALE	Coolie	P	3D	P	10Y	III	N	P	P	N	N	N	N	N	N	0.0607	P	0.4111	N	N
37	65	FEMALE	Home Maker	P	3D	P	4Y	III	N	N	N	P	N	P	N	N	N	0.0921	N	0.321	N	P
38	67	MALE	Farmer	P	4D	P	10Y	III	N	N	N	N	N	N	N	N	N	0.0538	P	0.6456	N	N
39	65	MALE	Coolie	P	3W	P	4Y	III	N	P	P	N	N	P	N	N	N	0.0477	P	1.1147	N	P
40	65	MALE	Farmer	P	1W	P	6M	III	N	P	P	N	N	P	N	N	N	0.0283	P	1.1574	N	P
41	55	MALE	Coolie	N		P	6Y	III	N	P	N	N	N	P	N	N	N	0.0596	N	0.0397	N	P
42	59	MALE	Coolie	P	2W	P	2Y	III	N	P	P	N	N	N	N	N	N	0.0348	P	0.9692	N	P
43	37	FEMALE	Home Maker	P	1W	P	6Y	III	N	N	N	N	N	N	N	N	N	0.1971	P	0.8503	P	P
44	45	FEMALE	Home Maker	P	5D	P	6M	III	N	N	N	N	N	N	N	N	N	0.0219	N	0.0736	N	P
45	53	MALE	Coolie	P	5D	P	20 Y	III	N	P	P	N	N	N	N	N	N	0.0498	N	0.2808	N	N
46	67	MALE	Driver	N		P	6M	III	N	P	P	P	N	P	N	N	N	0.023	P	1.0214	N	P
47	54	MALE	The Master	P	3D	P	3Y	III	N	P	P	N	N	P	N	N	N	0.0286	P	1.0543	N	P
48	48	MALE	Coolie	N		P	7Y	III	N	P	P	N	N	N	N	N	N	0.1349	P	1.1119	N	P
49	65	FEMALE	Home Maker	N		P	2Y	III	N	N	N	N	N	N	N	N	N	0.0361	P	1.116	N	P
50	72	MALE	The Master	N		P	2Y	III	N	P	N	N	N	N	N	N	N	0.034	P	0.7717	N	P